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Paternidad extra pareja y variación genética individual: implicaciones en la eficacia biológica del papamoscas cerrojillo, ***Ficedula hypoleuca***

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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La presente tesis explora en el papamoscas cerrojillo (*Ficedula hypoleuca*) los mecanismos que promueven la paternidad extra pareja, así como los efectos que este fenómeno tiene en los caracteres sexualmente favorecidos y en el éxito reproductivo de los individuos. Se evalúa además, el impacto de la variabilidad genética en la aptitud individual medida en base a la supervivencia. A lo largo de la tesis, se han desarrollado nuevos marcadores neutrales (microsatélites) y sometidos a selección (Complejo Principal de Histocompatibilidad), que permitirán abordar futuras cuestiones en ecología y/o evolución.

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A todo aquel con el que compartí penas

y alegrías durante estos años

Resumen

Las técnicas moleculares han revolucionado la forma de entender la ecología animal en general, y la de aves en particular. Su aplicación reveló la existencia de paternidad extra-pareja (EPP; de sus siglas en inglés) en aves, un descubrimiento considerado como el más importante en el campo de los sistemas de emparejamiento aviar de las últimas décadas. Hoy, se sabe que en más del 70% de las especies de aves estudiadas existe paternidad extra pareja (poligamia genética) pero los mecanismos que ocasionan este fenómeno están aún lejos de ser comprendidos. En esta tesis investigamos qué factores promueven la evolución y mantenimiento de la EPP en el papamoscas cerrojillo (*Ficedula hypoleuca*), un paseriforme con dimorfismo sexual y una estrategia reproductiva mixta, esto es, principalmente monógamo pero en el que tanto la poligamia social como vía extra pareja son relativamente comunes. En la población de estudio, la mayoría de las hembras tuvieron EPP con machos ya emparejados (cuyas hembras sociales ya no eran fértiles) y de mayor tamaño, con plumajes dorsales más negros y manchas frontales mayores que las respectivas parejas sociales. Ser polígamo (social o vía extra pareja) redundó en un mayor éxito reproductivo anual, pero las probabilidades de serlo no fueron constantes a lo largo de la temporada de cría, sino que disminuyeron con el avance de ésta. Nuestros resultados sugieren por tanto, que la selección sexual debe potenciar la evolución de los caracteres seleccionados en contextos extra pareja, así como ser la principal responsable de la llegada temprana de los machos con respecto a las hembras tras la migración primaveral. A nivel individual, la mayoría de los eventos extra pareja ocurrieron durante los periodos de puesta o incubación de la hembra social del macho extra pareja, a pesar del gran número de hembras fértiles presentes en la población antes y después de esos periodos. Parece existir así una estrategia por parte de los machos consistente en guardar a la hembra social durante su pico de fertilidad y buscar EPP después. La variabilidad genética, medida como la heterocigosidad calculada con marcadores neutrales, no estuvo

relacionada con la probabilidad de sobrevivir hasta el reclutamiento como reproductor ni a lo largo de los subsiguientes años. Esta falta de correlación fue independiente de la fenología de la reproducción, la carga parasitaria o el estatus de la nidada. Se ha sugerido que la heterocigosidad medida con marcadores neutrales puede ser una pobre aproximación a la diversidad genética de los individuos y que ésta debería medirse en genes funcionales. Por ello, en esta tesis se desarrolla un protocolo que permite el diseño de cebadores específicos del Complejo Principal de Histocompatibilidad (MHC), clase II. Este es un complejo de genes que está involucrado en la respuesta inmunitaria y, por tanto, bajo fuertes presiones selectivas, pero cuya caracterización hasta la fecha ha sido complicada en el orden de los Paseriformes debido al alto número de duplicaciones génicas que suele presentar.

Introducción

La biología molecular ha ido incrementando su relevancia en los estudios de ecología durante las últimas décadas hasta convertirse hoy en una herramienta prácticamente indispensable. El desarrollo de la PCR (reacción en cadena de la polimerasa) en los años 80 (Mullis y col. 1986), el progresivo descubrimiento de nuevos marcadores moleculares (isoenzimas, microsatélites, SNPS etc.) y el abaratamiento de los costes han permitido abordar múltiples cuestiones en ecología del comportamiento, biología evolutiva y de la conservación (ej. Andersson 1994; Hedrick y Kalinowski 2000).

El tremendo impacto que el auge molecular ha tenido en nuestro conocimiento queda reflejado en los estudios sobre la elección de pareja. Cuestiones como qué beneficios conlleva el discriminar unos individuos respecto a otros a la hora de emparejarse y qué consecuencias evolutivas tiene para ambos sexos, han sido y siguen siendo preguntas candentes en biología evolutiva y de la conducta (ej. Andersson 1994; Jennions y Petrie 1997; Mays and Geoffrey 2004). Tradicionalmente, el éxito reproductor de los machos se ha medido en base al número de parejas sociales y/o prole sacada a lo largo de la temporada de cría. Sin embargo, los estudios de paternidad han demostrado que multitud de especies monógamas tienen paternidad extra pareja (revisado en Griffith y col. 2002; Coleman y Jones 2011). Esto da lugar a sistemas genéticamente polígamos, que incrementan la varianza en el éxito reproductor entre individuos y potencian las presiones selectivas sobre los rasgos sexualmente seleccionados (Webster y col. 1995). Asimismo, gracias a las técnicas moleculares, es posible examinar los posibles beneficios genéticos derivados de la elección de pareja (Mays y Geoffrey 2004; Akçay y Roughgarden 2007). Esto no solo es clave en biología evolutiva, sino también en otros campos como el de la biología de la conservación puesto que, actualmente, es posible cuantificar el grado de parentesco entre individuos o el impacto de la endogamia en la eficacia biológica y el efecto que

ello tiene en el declive de las poblaciones (Hedrick y Kalinowski 2000; Keller y Waller 2002).

El fenómeno de la paternidad extra pareja

En una gran variedad de especies y taxones (peces, reptiles, mamíferos, aves) las hembras son fertilizadas por más de un macho, dando lugar a descendencia con paternidad mixta (Griffith y col. 2002; Liebgold y col. 2006; Cohas y Allainé, 2009; Coleman y Jones 2011). En aves, la confirmación de éste fenómeno en los años 70 supuso un cambio de paradigma en el estudio de los sistemas de emparejamiento. Tal es el caso, que el prestigioso ornitólogo David Lack es recordado a menudo por concluir en 1968, tras una minuciosa revisión, que más del 90% de las subfamilias de paseriformes estudiadas hasta la fecha eran monógamas. La revisión que Griffith y col. (2002) realizaron unas décadas después, una vez que los estudios de paternidad se popularizaron, mostró un panorama muy diferente al detectarse EPP en un 86 % de las aves paseriformes socialmente monógamas.

Numerosos estudios han examinado las causas que promueven la evolución de la EPP en aves (ej. Griffith y col. 2002; Westneat y Stewart 2003; Arnqvist y Kirkpatrick 2005; Akçay y Roughgarden 2007). Los beneficios de este fenómeno parecen claros en el caso de los machos, ya que éstos pueden aumentar su éxito reproductivo sin coste alguno en inversión parental. En este sentido, son abundantes los trabajos que muestran que la incidencia de EPP no se distribuye al azar entre los machos de la población, sino que está asociada con ciertos rasgos masculinos (ej. ornamentación: Cordero y col. 1999, Bitton y col. 2007; edad: Lubjuhn y col. 2007). Pero el hecho de que unos individuos ganen paternidad implica, lógicamente, que otros la pierdan. Así, en tanto que la paternidad ganada fuera del nido no se vea contrarrestada por la perdida en el nido social, la varianza en el éxito reproductor de

los machos incrementará y, con ello, la presión selectiva sobre los rasgos que predicen el éxito en la EPP (Webster y col. 1995).

En cuanto a las hembras, los beneficios que este comportamiento puede aportar no son tan obvios ya que ellas no pueden aumentar su descendencia al copular con más machos. En las especies monógamas, especialmente en las migratorias, la elección de pareja por parte de las hembras está limitada por el número de machos sin emparejar, por el de hembras buscando emparejarse y por los costes de búsqueda, variables que dependen de la fenología de cría. El resultado es que no todas las hembras tienen la oportunidad de emparejarse con el individuo preferido. Así, se sugiriere que la EPP puede ser una estrategia para sobreponerse a las limitaciones de la elección de pareja social. Los beneficios de tipo directo, como alimentación o protección, se asume que están cubiertos por la pareja social (Jennnions y Petrie 2000; Akçay y Roughgarden 2007; ver, sin embargo, Gray 1997). Por ello, se piensa que el beneficio obtenido debe estar relacionado con la calidad genética de la descendencia, lo que repercutirá, en última instancia, en la eficacia biológica de las hembras. Las hembras podrían así incurrir en copulas extra pareja (EPCs), tratando de maximizar la diversidad genética de la prole, buscando machos genéticamente más compatibles, de mayor calidad genética o para protegerse ante la posible infertilidad de su pareja social (Tabla 1; Griffith et al 2002.). Sin embargo, las evidencias que demuestran, de forma concluyente, alguno(s) de los beneficios arriba citados (ej. Gerlach y col. 2011) son escasas, siendo la mayoría de tipo correlativo (revisado en Akçay y Roughgarden 2007). Así, por ejemplo, la relación positiva entre rasgos fenotípicos masculinos (indicadores de calidad) y el éxito en EPP encontrada en muchos estudios (ej. Cordero y col. 1999, Bitton y col. 2007), podría deberse a preferencias femeninas o, por el contrario, ser una simple consecuencia de la mayor inversión en EPCs realizada por los machos en mejor condición física. Esta falta de apoyo empírico, unida a los costes que puede acarrear a las hembras copular fuera de la pareja (ej. disminución del cuidado parental por parte del macho social), ha llevado a algunos autores a sugerir

que este comportamiento no es adaptativo para las hembras y que simplemente se da porque, debido el acoso y agresividad de los machos, rechazar las EPCs es más costoso que aceptarlas (Arnqvist y Kirkpatrick 2005). Por tanto, el problema de si el comportamiento extra pareja ha evolucionado primariamente como una táctica masculina o femenina es aún un tema ampliamente debatido en la literatura (revisado en Westneat y Stewart 2003; Eliassen and Kokko 2008).

Una cuestión estrechamente ligada a lo adaptativo o no de este fenómeno es la gran variación en los porcentajes de prole extra pareja que existe entre especies, entre diferentes poblaciones de la misma especie e, incluso, entre años dentro de una misma población. Por poner un ejemplo, en una misma población de pechiazules (*Luscinia s. svecica*) el porcentaje de pollos extra pareja varió entre años del 7 al 33% (Johnsen y Lifjeld 2003). Mientras que la variación inter-específica parece tener un componente filogenético elevado (Arnold y Owens 2002), las diferencias dentro de especies y/o años dentro de una población parecen estar supeditadas a factores ecológicos que determinan el balance entre los costes y los beneficios de incurrir en paternidad extra pareja (Arnold y Owens 2002; Griffith y col. 2002). Hay que recordar que una relación extra pareja emerge de un conflicto de intereses entre una hembra, el macho social y el macho extra pareja. Por ejemplo, una alta sincronía de cría puede permitir a las hembras comparar más eficientemente la calidad de los candidatos extra pareja mientras que, para los machos, aumentaría las posibilidades de tener EPP debido al mayor número de hembras fértiles existentes (Stutchbury y Morton 1995). Sin embargo, la sincronía puede variar entre individuos (individuos que crían temprano y tarde son asincrónicos respecto al grueso poblacional) e igualmente lo pueden hacer las estrategias que éstos siguen. Por ejemplo, ante una alta sincronía, los machos de alta calidad podrían aumentar su inversión en la búsqueda de EPCs, mientras que los de baja calidad podrían hacerlo en la protección de sus hembras sociales para evitar perder paternidad (Stewart y col. 2006). Por lo tanto, para entender cómo ha podido evolucionar la EPP es necesario comprender el comportamiento a

nivel individual y cómo las estrategias seguidas por los individuos varían en función de las oportunidades de paternidad adicional y el riesgo de pérdida de paternidad. Todo ello requiere conocer en cierto detalle la distribución espacio temporal de los individuos en la población (Wesneat y Stewart 2003; Stewart y col. 2006).

Efecto de la variabilidad genética sobre la eficacia biológica

Desde hace siglos se ha visto que cuando individuos emparentados se reproducen, su descendencia normalmente sufre costes en viabilidad. Un ejemplo clásico al respecto son las dinastías reales europeas. Recientemente, mediante genealogía, se ha confirmado que el último representante de los Austrias en España, el rey Carlos II, que padecía de cierto retraso mental, continuas enfermedades e impotencia, tenía una alta carga endogámica consecuencia del incesto recurrente entre sus antepasados (Álvarez y col. 2009). Dejando a un lado a humanos, la relación entre eficacia biológica y diversidad genética tiene fuertes implicaciones en contextos de producción animal, conservación o evolución (revisado en Hedrick and Kalinowski 2000; Hansson and Westerberg 2002; Keller and Waller 2002; Coltman and Slate 2003; Kempenaers 2007). Sin embargo, cuando se trabaja con poblaciones naturales, la reconstrucción de genealogías resulta complicada, si no imposible, debido a la falta de información sobre los individuos fundadores o la incidencia de eventos extra pareja (Keller y Waller 2002). Por esta razón, el uso de marcadores genéticos como una aproximación a la diversidad genética individual (heterocigosidad) y su posterior correlación con aspectos relativos a la eficacia biológica (HFC), se ha popularizado en las últimas décadas (revisado en Coltman y Slate 2003; Chapman y col. 2009).

Varios son los mecanismos que pueden causar una relación entre heterocigosidad y aspectos afines a la eficacia biológica, como resistencia a los parásitos (Westerdahl y col. 2005), supervivencia (Da Silva y col. 2006) o éxito reproductor (Hanson y col. 2001). La relación con la eficacia biológica puede surgir de

forma directa, cuando la heterocigosidad en los propios marcadores causa el efecto, vía super-dominancia (Szulkin y col. 2010). Este mecanismo puede ser importante cuando la heterocigosidad se mide con marcadores funcionales (aloenzimas o Complejo Principal de Histocompatibilidad), pero es difícil de conciliar con el uso de marcadores neutrales, como son generalmente los microsatélites (Jarne and Lagoda 1996). En este caso, las HFCs surgen de forma indirecta; esto es, los loci usados pueden estar asociados con otros que influyen en el rasgo medido, o bien reflejar la variabilidad genética individual a nivel del genoma (Szulkin y col. 2010).

Tanto estudios teóricos como empíricos sugieren que la varianza en eficacia biológica explicada por las HFCs es baja, no superando el 3.6% (Chapman y col. 2009). A pesar de ello, son varias las circunstancias que pueden intensificar la relación entre heterocigosidad y eficacia biológica; principalmente, las características de la población, las del rasgo medido, y las condiciones en las que éste es medido (Slate y col. 2004; Szulkin y col. 2010). Poblaciones con emparejamientos de consanguineidad, deriva genética, cuellos de botella recientes o la mezcla de poblaciones, son factores que crean desequilibrio de identidad (correlación de heterocigosidad entre loci) y favorecen la aparición de HFCs (Lynch and Walsh 1998; Szulkin y col. 2010). Asimismo, la medición de rasgos estrechamente relacionados con la eficacia biológica y con arquitectura genética compleja (afectados por muchos loci; supervivencia, fertilidad o éxito reproductivo a lo largo de la vida), favorecerá la detección de HFCs (Coltman y Slate 2003; Szulkin y col. 2010). Por el contrario, una relación entre heterocigosidad y aptitud será más difícilmente detectable cuando aquella se mide en base a caracteres morfológicos o de comportamiento, que suelen estar sometidos a selección estabilizadora (Coltman y Slate 2003). Por último, las condiciones de estrés podrían intensificar la relación entre heterocigosidad y eficacia biológica si, por ejemplo, los individuos con mayor diversidad alélica son capaces de hacer frente a una mayor diversidad de condiciones ambientales (Chapman y col. 2009).

El papamoscas cerrojillo como modelo de estudio

El Papamoscas Cerrojillo (*Ficedula hypoleuca*), es uno de los paseriformes mejor estudiados a lo largo de Europa (revisado en Lundberg y Alatalo 1992 y Morales 2012). Ave de pequeño tamaño (11-14 gr. de peso) y notable dimorfismo sexual durante la época de cría, es un migrante transahariano que durante la época de cría se distribuye ampliamente por el Parleártico occidental (Europa occidental, Oriental y suroeste de Siberia) y cuyas áreas de invernada se localizan en la zona subtropical del oeste de África (Lundberg y Alatalo 1992). En época reproductiva, habita bosques húmedos, preferentemente de robles y coníferas, donde cría en los agujeros naturales de los árboles. La especie tiene, sin embargo, una fuerte predilección por las cajas nido lo que, junto a la alta tolerancia que muestra a la manipulación, hacen de ella un modelo ideal para el estudio científico.

Macho y hembra de papamoscas cerrojillo. Fotos: David Canal

Sin duda, uno de los mayores atractivos de la especie es la gran variación fenotípica existente, especialmente, en los caracteres sexuales secundarios de los machos a nivel intra e inter poblacional. Mientras que las hembras son de color pardo-grisáceo, el color del plumaje en machos varía a lo largo de un continuo, desde un plumaje completamente negro hasta un marrón similar al de las hembras. Los

primeros predominan en las poblaciones noruegas e ibéricas, mientras que plumajes más marrones son comunes en las poblaciones centro-europeas (Lundberg y Alatalo 1992; Lehtonen y col. 2009). Varios estudios han demostrado que los individuos con plumaje más negro son preferidos por las hembras en poblaciones escandinavas (Järvi et al. 1987; Saetre et al. 1994) e ibéricas (Galvan y Moreno 2009), tienen repertorios de canto más variados (Lampe y Espmark 1994) y ceban más a sus pollos que los machos más marrones (Sætre y col. 1995). Todo ello, ha llevado a sugerir que el plumaje marrón es característico de machos en pobre condición física, que ante el alto coste energético que supone la muda primaveral, mantendrían ciertas zonas con el plumaje de invierno (Rohwer y Butcher 1988). Alternativamente, poseer un plumaje parecido al de las hembras podría ser adaptativo en individuos de baja calidad ya que, al sufrir éstos menos agresividad por sus conespecíficos, podrían criar en territorios de superior calidad de los que les correspondería (Slagsvold y Saetre 1991; Saetre y Slagsvold 1992). Dado el componente heredable del carácter (Slagsvold y Lifjeld 1992), la preferencia femenina por plumajes oscuros contrasta con la predominancia del plumaje marrón en centro-Europa. Allí, el papamoscas cerrojillo convive en simpatría con la especie hermana, el papamoscas collarino (*F. albicollis*), con la que puede hibridar, de forma que, en esas poblaciones, se piensa que el plumaje marrón actúa como señal de reconocimiento intra-especifico para las hembras de cerrojillo que prefieren éste respecto a ornamentos más oscuros, típicos de collarino (Sæther y col. 1997). Otro de los rasgos que varía ampliamente entre poblaciones es el tamaño de la mancha blanca frontal de los machos, siendo pequeño en las poblaciones noruegas en comparación con la que poseen los individuos de las poblaciones ibéricas (Dale y col 1999). Mientras que en las primeras el carácter no parece ser importante en la elección de pareja, en las poblaciones ibéricas el rasgo es heredable (Potti y Canal 2011) y favorecido por selección sexual al ser los machos con manchas mayores preferidos por las hembras a la hora de aparearse (Potti y Montalvo 1991). Asimismo, se ha demostrado experimentalmente que las hembras ponen huevos de menor

tamaño cuando sus parejas poseen manchas de pequeño tamaño (Osorno y col. 2006). Además de la variación fenotípica, las poblaciones europeas también presentan diferencias a nivel genético, ya que análisis con microsatélites y ADN mitocondrial indican que las poblaciones ibéricas están altamente diferenciadas del resto de poblaciones europeas, las cuales a su vez muestran cierta divergencia en función de la población y/o marcador estudiado (Haavie y col. 2000; Lehtonen y col. 2009).

Puesta típica de papamoscas cerrojillo con 5 huevos y pollo a los 13 días de edad.
Foto: Inés Valencia y Carlos Camacho

El papamoscas cerrojillo es principalmente monógamo. Tras la migración, los machos llegan antes que las hembras a las áreas de cría (Potti y Montalvo, 1991b), localizan una cavidad, la defienden ante otros machos e intentan atraer a una hembra. Algunos machos son polígamos politerritoriales y después de emparejarse ocupan una segunda oquedad donde crían con otra hembra (Lundberg y Alatalo 1992). Tal sistema social, en primer lugar, y el descubrimiento de paternidad extra-pareja dentro de la especie después, han hecho que la ecología reproductiva de esta especie haya sido ampliamente estudiada a lo largo de las últimas décadas. Estudios previos han profundizado en los posibles comportamientos que dan lugar a la poliginia (hipótesis del desconocimiento (Slagsvold y Lifjeld 1997) y engaño (Alatalo et al. 1981)) y en los costes de ser polígamo en machos y pasar más tiempo alejado del nido durante el

periodo fértil de la hembra (Björklund y Westman 1983, Björklund et al. 1992, Westneat 1994) frente a los beneficios que este estatus reporta (Birkhead y Møller 1992). Asimismo, en hembras, se han investigado los costes que supone emparejarse con machos polígamos (Huk y Winkel 2006) y los comportamientos desarrollados para intentar evitar que el cónyuge se convierta en polígamo (agresividad con otras hembras; Slagsvold y Saetre, 1991; Slagsvold et al. 1992, Rätti 1999). En el ámbito extra pareja, se han explorado los efectos de la densidad poblacional en las tasas de EPP dentro y entre poblaciones (Lifjeld et al. 1991; Gelter y Tegelström 1992; Rätti et al. 2001), los comportamientos desarrollados por parte de los machos para evitar perder paternidad (custodia de la pareja; Björklund y Westman 1983; Alatalo y col. 1987), los costes que para las hembras tiene la disminución del esfuerzo parental por parte del cónyuge ante la sospecha de haber sido engañado (Lifjeld et al. 1998) y los rasgos que influyen en el éxito o perdida de paternidad (Lifjeld et al., 1997; Lehtonen et al., 2009; Rätti et al., 1995; Moreno et al., 2010). A pesar de tales esfuerzos, son comunes los resultados contradictorios entre estudios. Así, todavía quedan cuestiones sin resolver referentes al papel del fenotipo masculino en el éxito extra-pareja, las causas de la variación entre años y poblaciones en el porcentaje de pollos extra pareja, o los costes y/o beneficios a largo plazo que tiene seguir una u otra estrategia reproductiva (poligamia y paternidad extra pareja) en ambos sexos.

OBJETIVOS Y ESTRUCTURA DE LA TESIS

Los objetivos principales de la tesis son: 1. Determinar qué circunstancias promueven la evolución y mantenimiento de la EPP en el papamoscas cerrojillo. 2. Evaluar la relación entre la diversidad genética (medida con microsatélites) y la supervivencia, un factor íntimamente ligado a la eficacia biológica individual.

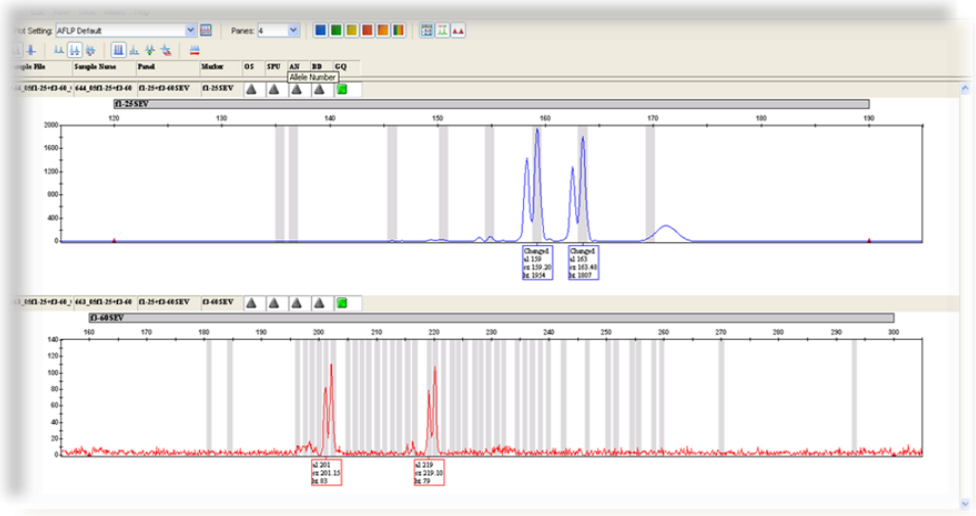
Para responder esas cuestiones, en la tesis se combina la información obtenida a partir del uso de marcadores neutrales (microsatélites) con la generada durante intensas campañas de trabajo de campo. La tesis se divide en 6 capítulos, cuyo desarrollo ha ido ligado, en gran medida, al de las herramientas moleculares necesarias para contestar a las cuestiones que se plantean. **En el capítulo I** se desarrollan nuevos marcadores neutrales específicos para la especie puesto que ni el número, ni la calidad de los existentes por entonces era el adecuado para los objetivos a realizar. Estos marcadores se utilizarán en los **capítulos II, III, IV** para explorar qué factores fomentan la obtención y/o pérdida de paternidad, principalmente, debido a EPCs (aunque la poligamia social se trata en el **capítulo III**). En concreto, en el **capítulo II** se investiga el papel del fenotipo en la EPP, el impacto de este fenómeno en el éxito reproductivo de los machos y las consecuencias que ello puede tener en la evolución de los caracteres sexualmente seleccionados. ¿Son más atractivos los individuos que ganan EPP y menos los que la pierden? ¿Ganar EPP aumenta las probabilidades de perder paternidad en el propio nido? son algunas de las preguntas que se intentan responder en este capítulo. Sin embargo, reducir el éxito o fracaso de ganar EPP al fenotipo puede ser algo simplista. Si bien, el fenotipo del macho puede determinar en última instancia la predisposición o rechazo de una hembra a copular (asumiendo que son los machos quienes buscan activamente EPCs) serán los factores espacio-temporales los que limiten el encuentro entre individuos y, por tanto, en primera instancia, la probabilidad de ocurrencia de las relaciones extra pareja. Esto es fácil de entender si pensamos en las posibilidades de tener un affaire (ganar paternidad) con

nuestra actriz de Hollywood favorita. En el mejor de los casos serán muy bajas, simplemente, porque ni espacial ni temporalmente coincidimos con ellas. Da igual si tiene muchos pretendientes (sincronía de cría) o si son muy ricos y atractivos (fenotipo) ya que, sencillamente, no estamos allí. Afortunadamente, las probabilidades de que nuestra pareja tenga tal affaire (perder paternidad) con esos mismos pretendientes, ricos y atractivos, son también bajas. Algo tan aparentemente intuitivo se ignora a menudo en los estudios sobre paternidad extra pareja, pero es de vital importancia si tenemos en cuenta que muchas aves son fértiles únicamente durante 5-10 días al año. Esos factores son explorados en el **capítulo III y IV**. En el **capítulo III** investigamos cuáles son las fuerzas selectivas que fomentan la llegada temprana a las áreas de cría de los machos respecto a las hembras tras la migración prenupcial. Los machos que llegan antes, ¿aumentan sus probabilidades de ser polígamos (sociales y/o extra pareja)?, ¿Obtienen mejores territorios?, ¿Cuál es el impacto que tiene llegar temprano a las áreas de cría sobre el éxito reproductivo de los machos? En el **capítulo IV** intentamos comprender la variación poblacional en los patrones extra pareja a través del entendimiento de la conducta individual. Analizaremos las estrategias que siguen los individuos (guardar el nido vs buscar cópulas extra pareja) en relación a las circunstancias que los rodean (ej. estado fértil de la pareja social, número de hembras extra pareja accesibles). Además, veremos cómo los patrones extra pareja pueden variar, en función de si éstos son analizados a nivel poblacional o individual (considerando únicamente la escala espacio-temporal en la que tienen lugar las interacciones). En el **capítulo V**, los marcadores aislados en el **capítulo I** junto a otros publicados por Leder y col. (2008) para la especie, son utilizados como indicadores de la diversidad genética del individuo. El principal objetivo es investigar, bajo distintos contextos (ej. carga parasitaria del nido, variación temporal en las fechas de eclosión), la influencia de la diversidad genética en la supervivencia de los individuos hasta el reclutamiento como reproductores y durante la etapa adulta subsiguiente. Sin embargo, tanto trabajos teóricos como empíricos (ej. Slate y col.

2004, Balloux y col. 2004, Chapman y col. 2009), sugieren que los marcadores neutrales pueden ser una aproximación relativamente pobre a la diversidad genética del individuo, y que estudiar la variabilidad genética en base a marcadores bajo selección sería un importante avance. Por ello, el último **capítulo (VI)** marca la senda a seguir en un futuro, esto es, estudiar la eficacia biológica en relación a la variación genética sujeta a selección. En este capítulo se desarrolla un protocolo que permite el diseño de cebadores específicos para la caracterización en papamoscas del Complejo Principal de Histocompatibilidad (MHC), un complejo de genes que juega un papel esencial en la respuesta inmunitaria. Además, se demuestra su utilidad para el grupo de los paseriformes en general, un orden donde el estudio del MHC ha entrañado tradicionalmente una gran dificultad debido al alto número de duplicaciones génicas que el MHC suele tener.

CAPÍTULO I

Polymorphic microsatellite markers isolated from a southern European population of pied flycatchers (*Ficedula hypoleuca iberiae*)



Electroferograma de dos microsatélites pertenecientes a un individuo heterocigoto

Canal D, Dávila JA, de Nova PJC, Ferrero E, Potti J. 2009. Polymorphic microsatellite markers isolated from a southern European population of pied flycatcher (*Ficedula hypoleuca iberiae*). Mol Ecol Res. 9:1375-1379

http://onlinelibrary.wiley.com/doi/10.1111/j.1755-0998.2009.02746.x/abstract

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ABSTRACT

Nine polymorphic microsatellite loci for the pied flycatcher (*Ficedula hypoleuca*), from a wild population in Spain, are isolated and its variability described on 70 individuals. The number of alleles per locus ranged from 6 to 41 and observed heterozygosity ranged from 0.75 to 0.98. These markers are being used to study mating strategies in *Ficedula hypoleuca iberiae*.

Keywords: Ficedula hypoleuca iberiae, microsatellites, molecular marker, primers.

INTRODUCTION

Three subspecies are currently recognized for the pied flycatcher (*Ficedula hypoleuca*): *F. h. hypoleuca* breeding from southern France to northern Europe, *F. h. sibirica* distributed across southwest Siberia and *F. h. iberiae* in the Iberian Peninsula (Sætre et al. 2001; Dickinson 2003).

Over time, pied flycatcher subspecies have been exposed to different pressures and responded in different ways, diverging phenotypically and genetically due to selection, genetic drift and mutation. Thus, the three subspecies present differences in traits as plumage colour, size of the forehead patch (Lundberg and Alatalo 1992) or song (Haavie et al. 2004) whereas at the genetic level Spanish pied flycatchers differ significantly from Czech and Norwegian ones at both microsatellite loci and mtDNA sequences (Haavie et al. 2000). Therefore, as a consequence of evolutionary processes members of different populations, subspecies or species will show higher degrees of genetic divergence with increasing time from isolation. As shown in the the chapter II most of the six microsatellites already published by Scandinavian researchers (Ellegren 1992; Primmer et al. 1996) showed some type of problem in Spanish pied flycatchers. Null alleles were detected in FhU1 and FhU3 whereas FhU4 showed weak polymerase chain reaction (PCR) amplifications. Regarding FhU5 and FhU6, PCR amplified a high number of unspecific bands, preventing their optimization. In addition, FhU1, FhU2, and FhU3 exhibited moderate levels of polymorphism (3, 6, and 7 alleles in 80 individuals, respectively). Therefore, development of additional, more informative microsatellite markers is required even in this relatively well-studied species. Recently, a new set of markers from a Finnish (*F. h. hypoleuca)* population has been published (Leder et al. 2008). Here we describe the development and optimization of a new set of highly variable, polymorphic microsatellite DNA loci isolated from a population of *F. h. iberiae*.

MATERIAL AND METHODS

Blood samples were collected from a wild population in La Hiruela (central Spain) and stored at room temperature in 100% ethanol. Genomic DNA was extracted from blood by a standard phenol-chloroform method (Sambrook et al. 1989). An enriched microsatellite genomic library was constructed following procedures modified from Hamilton et al. (1999). Briefly, 50 ug of DNA were digested with 100 U each of *Nhe*I, *Bsu*RI (*Hae*III) and *Rsa*I (Fermentas). Restriction fragments of 300 - 1000 base pairs were excised from agarose gel, blunt ended, dephosphorylated and ligated to SNX linkers. Microsatellite enrichment was carried out by hybridization capture of repeat sequences using biotinylated $(CAT)_8$, $(GAT)_8$, $(GACA)_7$, $(GATA)_7$, $(TCCA)_7$ and $(TGGA)_7$ oligonucleotides and streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen) and subsequently amplified by PCR with SNX-F primer. Amplified DNA was ligated into the XbaI site of pUC19 (Fermentas) and plasmid constructs were used to electroporate ElectroTen-Blue electroporation-competent *Escherichia coli* cells (Stratagene). Positive recombinants were replated on LB agar medium and lifted onto nylon membranes, probed with DIG-labelled microsatellite motives used in enrichment. Seventy positive clones were sequenced in ABI 3130xl Sequencer (Applied Biosystems).

Sequences were edited in BIOEDIT 7.0.5.2 (Hall 1999) and primers for those sequences containing microsatellites were designed by eye. PCRs were performed in 10-μL reaction volumes (BIOTOOLS: 1x standard reaction buffer, 2.0 mM MgCl2, 0.2 mM of each dNTP and 0.5 U Taq Polymerase) using 0.5 μM of each primer and containing 50 ng of DNA as template. PCR amplifications consisted of initial denaturation (2 min at 94°C) followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (Table 1) and 30 s at 72°C, plus a final extension of 10 min at 72°C. One primer of each pair that reliably amplified a polymorphic locus was tagged with VIC, FAM, PET or NED fluorescent labels (Applied Biosystems). Loci from seventy

individuals were genotyped on ABI PRISM 3130xl sequencer (Applied Biosystems). Allele sizes were determined with Genescan 600-LIZ internal size standard and Genemapper 4.0 software (Applied Biosystems).

Observed and expected heterozygosities were calculated using ARLEQUIN (Excoffier et al. 2005), which was also used to analyse linkage disequilibrium and assess the significance of deviations from Hardy–Weinberg equilibrium (100 000 Markov chains).

Nine loci remained after others were discarded because of sequence redundancy, lack of microsatellite repeat, insufficient flanking sequence for primer design, monomorphism or non-specific PCR products. The nine loci were highly variable: all except one had 10 or more alleles per locus and, remarkably, two of them displayed more than 40 alleles per locus. Observed heterozygosity ranged from 0.75 to 0.98. All loci conformed to Hardy-Weinberg equilibrium and no pairs of loci showed significant linkage disequilibrium (Table 1).

Microsatellites here developed have been successful in revealing high rates of extra-pair paternity in relation to patterns of sexual selection on male secondary sex traits in our study population (Chapter II). Overall, these markers increase genetic resources for the species and, due to their high variability, are a useful tool for a wide variety of purposes, as studies of genetic diversity, breeding strategies or population structure.

Table 1. Summary data for nine microsatellite loci isolated from the pied flycatcher with primer sequences, number of individuals genotyped (N), annealing temperature in PCR (Ta), repeat motif of the cloned microsatellite, GenBank Accession number, number of alleles (A.), size range (base pairs) of observed alleles, observed heterozygosity (H_O), expected heterozygosity (H_E) and *P* value of the test to detect significant departure from Hardy-Weinberg equilibrium. N (NED), F (FAM), V (VIC) and P (PET) indicate the fluorescent label used. Polymorphism data are based on adults from a single population in central Spain.

Locus	Primer sequence (5'-3')	N	Ta (ºC)	Repeat motif	GenBank no.	A	Size range	H_O	H_E	*P*
Fhy 1-25	F^F: TGGCAGGAGTAACCCAGATG R: CAAACATCCACACCTGACTG	70	54	(CTGT)10	FJ389732	6*	136 - 169	0.75	0.70	0.96
Fhy 3-60	F^P: TTCTTTACGGCTCTGCATTG R: CAGGAAAGTGCCCAGCAATC	70	52	(CCAT)16	FJ389733	19*	181 - 273	0.98	0.92	0.99
Fhy 3-85	F^V: GTGACAACTGAGCAAGAATTCC R: TGCTGCTCTCAGATGGTTCTTC	70	63	(GGAT)14	FJ389734	17	219 - 315	0.92	0.86	0.66
Fhy 4-95	F^N: ATGTGGACACAAGAACATGG R: TGTGTATGTGTCCATCTCAG	70	56	(GGAT)15	FJ389735	14	152 - 216	0.81	0.90	0.25
Fhy 5-75	F^F: ACTAGTTCCGGCAGGGTATCCA R: CAATGTCCTGCACATGAAATGG	70	63	(CCAT)12	FJ389736	25	134 - 278	0.85	0.93	0.49
Fhy 6-126	F^F: GTTTTCTGTCTCCCTCAGGAC R: GGGTGTGACAAGTGTGTACAT	70	60	(TATC)43	FJ389737	34*	137 - 316	0.98	0.96	0.58
Fhy 9-98	F^N: AGCCCCAGACATTGAGATG R: TGATGCATGCCAGTGAATC	70	60	(CAT)16	FJ389738	14	121 - 166	0.93	0.91	0.21
Fhy 14-41	F^P: GATCACAAGTTGGACTTGATG R: CACCACATCTATTGCTGACAG	70	60	(TATC)16	FJ389739	10	184 - 228	0.84	0.87	0.59
Fhy B4-7	F^N: TGCAGGGATTCAGCAGGACT R: CCAATAACTGCAAGCACTGG	70	65	(TGA)97	FJ389740	41	295 - 667	0.93	0.96	0.25

CAPITULO II

Male phenotype predicts extra pair paternity in pied flycatchers

Ejemplos de manchas frontales y plumajes dorsales en los machos de papamoscas cerrojillo. Fotos: Inés Valencia y David Canal

Canal D, Potti J, Dávila JA. 2011. Male phenotype predicts extra pair paternity in pied flycatchers. Behaviour. 148:691-712.
http://www.ingentaconnect.com/content/brill/beh/2011/00000148/F0020005/art00006

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ABSTRACT

Extra-pair paternity has the potential to increase male reproductive success and in turn the potential for sexual selection to act on male traits predicting extra pair mate success. There is large variation among European populations of pied flycatchers (*Ficedula hypoleuca*) in the extent to which male traits predict success in extra pair mating behaviour. In an Iberian population with a relatively high proportion of extra-pair young multiple male traits were involved in extra pair paternity success. Cuckolder males had larger tarsi and more attractive sex ornaments (blacker dorsal plumage and larger forehead patches) than the individuals they cuckolded, results not replicated in other populations. Previous studies in the species have shown that all traits associated with achieving success in extra pair paternity in our population are heritable and likely reliable indicators of male quality. Siring additional young was an advantageous strategy for males as it did not imply loss of paternity in their own nests. Our results, thus, suggest that this behaviour may enhance the evolution of male traits associated to success in extra-pair paternity in this population.

Keywords: Extra-pair paternity, male phenotype, pied flycatcher, sexual selection.

INTRODUCTION

A large body of work has conclusively demonstrated in many vertebrates that social monogamy does not necessarily imply genetic monogamy (fish: Sefc et al. 2008; amphibians: Liebgold et al. 2006; mammals: Cohas and Allainé 2009; birds: Griffith et al. 2002). Although the reasons for female promiscuity are not obvious and are consequently much discussed (Dixon et al. 1994; Jennions and Petrie 2000; Arnqvist and Kirkpatrick 2005; Simmons 2005; Akçay and Roughgarden 2007; Griffith 2007; Mays et al. 2008), siring additional offspring outside the pair bond (extra-pair young; EPY) seems clearly adaptive for males by increasing their reproductive success while often taking advantage of the paternal care provided by other males (extra-pair paternity; EPP). Independent of its adaptive function, EPP may play an important role in the evolution of sexual characters when its distribution among males is non-random and heritable male traits predict EPP success (Webster et al. 1995). As long as EPP is not counterbalanced by paternity losses at the within pair level (Andersson 1994; Webster et al. 1995), EPP will increase the variance of male fitness and, in turn, the strength of sexual selection on traits predicting paternity success.

A number of studies have related either success in EPP or loss of paternity to male age (Bouwman et al. 2007; Lubjuhn et al. 2007), ornamentation (Bittton et al. 2007; Balenger et al. 2009), condition (Møller et al. 2003) or body size (Yezerinac and Weatherhead 1997; Hutchinson and Griffith 2008) though even in different populations of the same species conflicting results regarding which traits are important for females are commonly reported (Akçay and Roughgarden 2007). Different environmental conditions and specific female preferences may cause targets of sexual selection to diverge across populations (Endler 1977; Lande 1981; Schluter and Price 1993; Dale et al. 1999). Additionally, loss of trait variability caused by prolonged selection may cause differences in what traits are currently better signaling male quality across populations (Dale et al. 1999) and thereby in the criteria used by

females in partner choice (Endler and Houde 1995; Freeberg et al. 1999). Given the consequences EPP may have on the evolution of male traits, understanding the role of the male phenotype in this widespread phenomenon and measuring its impact on individual reproductive success are central tasks (Westneat and Stewart 2003; Albrecht et al. 2009).

The pied flycatcher (*Ficedula hypoleuca*) is a predominantly monogamous, hole nesting songbird. After mating, a number of males try to acquire a second female and some succeed in becoming socially polygamous (reviewed in Lundberg and Alatalo 1992). In addition to male age, sex traits such as mantle colour, wing and forehead patch sizes have been shown to function as quality indicators in pied flycatcher males and/or being sexually selected in social contexts in the species (Potti and Montalvo 1991b; Lundberg and Alatalo 1992; Slagsvold and Lifjeld 1992; Sætre et al. 1994; Lehtonen et al. 2009a) or in the closely related collared flycatcher *Ficedula albicollis* (Gustafsson et al. 1995; Ellegren et al. 1996; Sheldon et al. 1997; Pärt and Qvarnström 1997; Török et al. 2003). Previous studies have also highlighted genetic polygamy in the pied flycatcher (e.g. Lifjeld et al. 1991; Gelter and Tegelström 1992; Rätti et al. 1995, 2001; Brün et al. 1996). However, the evidence of EPP in relation to male traits is ambiguous, with some work associating variation in paternity to male secondary sex traits (Lifjeld et al. 1997a; Lehtonen et al. 2009a) and others failing to show such relationships (Rätti et al. 1995; Moreno et al. 2010). Several studies have shown both genetic and phenotypic differences (e.g. variation in plumage colour) between Iberian and northern European populations (e.g. Lundberg and Alatalo 1992; Haavie et al. 2000; Lehtonen et al. 2009b; Qvarnström et al. 2010). In the only study performed to date in Iberian pied flycatchers paternity loss within the own nest was unrelated to ornamentation but was associated to male age and corticosterone levels, whereas traits associated to EPP success were not analyzed (Moreno et al. 2010). Therefore, whether male phenotype (age, size and ornaments) affects EPP success and how the latter

influences male reproductive fitness and thereby sexual selection on male secondary traits are questions largely unresolved in these populations.

The aims of our study were (1) to examine whether male phenotype (age, size and ornaments) predicts extra-pair mating; and (2) to assess the impact of this behaviour on male reproductive success in a Spanish population of pied flycatchers. To this end, we used molecular tools to find EPY and the males who sired them. We then focus on which male traits are related to EPP and quantify how this behaviour affects male reproductive success.

MATERIAL AND METHODS

Field work

The study was done during the 2005 breeding season in a population of pied flycatchers breeding in nest-boxes in central Spain which has been monitored since 1984 (e.g. Potti and Montalvo 1991a, b; Potti and Canal 2011).

All nests were regularly checked to ascertain exact laying date, clutch size, hatching date and number of fledglings. Parent birds were captured with a nest-box trap while they were feeding 8-day-old nestlings. All adult birds were marked with a numbered metal ring and a unique combination of colour rings. Age of many breeding birds was known with precision due to high natal philopatry (Potti and Montalvo 1991a). Previously unringed birds were aged as first year (yearlings hereafter) or older on the basis of plumage traits (Potti and Montalvo 1991b; Lundberg and Alatalo 1992; Svensson 1992). Birds were weighed (to the nearest 0.1 g) and measured for tarsus length (to the nearest 0.01 mm), wing length (to the nearest 0.5 mm) and height and width of the forehead patch (to the nearest 0.01 mm). The primary feather where the white wing patch starts (counted descendently and looking at the outer feather vane) was also recorded to give an estimate of the (unrecorded in the study year) wing patch area (as the former predicts the latter: $R^2 = 54\%$; authors' unpubl. data). The area of

the forehead patch was calculated as patch height × width. In males, the percentage of black feathers in the back of mantle was visually estimated, high values indicating a black plumage (Potti and Montalvo 1991c; Lundberg and Alatalo 1992).

Nestlings were ringed, measured and weighed at the age of 13 days. Blood samples were taken from all individuals by puncturing the brachial vein and stored in ethanol.

Molecular methods

Individuals were genotyped at seven polymorphic microsatellite loci (Table 1). Four of these loci had been developed by Ellegren (1992) (fhu1 and fhu2) and Primmer et al. (1996) (fhu3 and fhu4) and the rest were developed by us (Chapter I). To further increase reliability in the assignment of genetic fathers we genotyped all individuals from nests containing young having mismatches with their putative father (see below) with three additional primers (fhy444, fhy466 and fhy310; Leder et al. 2008). PCR conditions followed the authors' recommendations.

Microsatellite markers were ran on an ABI PRISM 3130xl DNA sequencer (Applied Biosystems). Allele sizes were determined according to Genescan 500-LIZ size standard and Genemapper version 4.0 (Applied Biosystems). Our sample size was 121 nests containing 578 nestlings. Once genotyped, 8 nests were excluded from further analyses since it was impossible to determine either presence or absence of EPP in them. All these nests shared similar features: small brood size (2-3 nestlings) and unknown (i.e. unidentified) social father. In the remaining 113 nests, 5% of the nestlings (out of 560) were unsampled due to predation or mortality prior to sampling. Thus, final sample size was 743 individuals: 531 chicks and 212 adults (113 females and 99 males, 14 being polygynous). We also captured four males defending natural holes in trees but they did not sire any young within the nest-box population and, thus, were not further considered.

Paternity analysis

The parentage analysis was performed using the program CERVUS 2.0 (Marshall et al. 1998). The combined probability of exclusion for all loci, given that the mother's alleles are known, was >99.9% (Table 1). This is the probability averaged over all loci that a randomly chosen male in the population will not match the alleles found in offspring. Using a maximum likelihood method, CERVUS calculates the confidence of paternity through a simulation where allele frequencies, number of candidate parents (103, in our case), proportion of candidate fathers sampled from the population (0.8), percentage of loci typed (0.98) and sampling errors (0.01) are taken into account. The simulation estimates the delta values, i.e. the difference in LOD (natural logarithm of the likelihood ratio) scores between the first and second most likely father at a given confidence level in order to generate a list of the most likely sires for a given nestling. We based our paternity assignments on a 95% confidence level. After null alleles were taken into account (as both Fhu1 and Fhu3 loci showed significant heterozygote deficits relative to Hardy-Weinberg equilibrium), genotypes of all young matched with their putative mother, hence no case of intraspecific brood parasitism was confirmed. A nestling was considered as EPY if their social father was not in the list of most likely sires given by CERVUS, or if there was another male in the population with a better match than the putative father (i.e. with positive and higher LOD scores). We identified the extra-pair sire when a male had none or one mismatch (i.e. due to null alleles; it was checked manually) and a high LOD score for a given nestling. Some nestlings were considered EPY of unknown sires (i.e. unidentified in the field) since no candidate male showed good matches with them.

Table 1. Numbers of alleles (N) at microsatellites, polymorphic information content (PIC), observed and expected heterozygosities (H.obs and H.exp, respectively) and probability of exclusion (P.excl) in the pied flycatcher study population.

Locus	N	H.obs	H.exp	PIC	P.excl
Fhu1	3	0.534	0.607	0.523	0.686
Fhu2	7	0.711	0.704	0.651	0.548
Fhu3	7	0.472	0.556	0.53	0.643
Fhu4	21	0.863	0.891	0.88	0.217
Fhy6-126	42	0.956	0.958	0.955	0.087
Fhy1-25	7	0.758	0.731	0.692	0.491
Fhy3-60	21	0.944	0.927	0.922	0.148
Fhy310	13	0.873	0.872	0.858	0.259
Fhy444	16	0.888	0.878	0.865	0.248
Fhy466	12	0.849	0.832	0.811	0.331

The combined probability of exclusion for all loci was >99.9%.

Statistical analyses

To see which male traits are important in EPP contexts we ran two different groups of tests: at the population level and pair wise tests which directly compare cuckolder males with the social males they cuckolded. The determinants of gaining and losing paternity at the population level were explored through three steps. First, we examined the variables involved in paternity loss (by comparing males losing and not losing paternity). Then, we did the same with the variables involved in paternity gain (by comparing males gaining and not gaining paternity). In both cases we used generalized linear models with binomial distribution and logit link functions, wherein dependent variables (gaining/not gaining EPP or losing/not losing EPP) were coded as 0/1 and laying date, male age, size and plumage traits were held as explanatory variables. The

link between gaining and losing paternity was examined with a 2 × 2 chi square goodness-of-fit test. In all these analyses we used data from both monogamous and primary nests of polygamous males after having previously confirmed the absence of differences in fledgling success between both types of nests ($\chi^2_1 = 1.53$, $p = 0.21$). Data from secondary broods of polygamous males were not considered in analyses to avoid pseudo-replication and because their status could affect paternity of the offspring if males spend less time potentially guarding them during the fertile period (Lundberg and Alatalo 1992). Wing length and the primary feather where the wing patch begins vary with age and were standardized before they were entered into the models. The probability that an EPP event occurred was also modelled as a function of the breeding date and the female breeding status of the social nest (monogamous, primary or secondary female) with a generalized linear model (binomial distribution and logit function).

Matched comparisons of traits and breeding dates between the cuckolder male(s) and the social male they cuckolded were done with pairwise t -tests. Due to non normal data distribution, age and plumage colour were analyzed with non-parametric, matched-pairs Wilcoxon tests. As in the models above, wing length and the primary feather where the wing patch begins were standardized by age before analyses. In three nests, young were fathered by two extra-pair males and we used the averages of both males. Data from secondary nests of polygamous males with EPP were not considered in analyses.

The impact of gaining or losing extra pair paternity on male total genetic reproductive success (number of fledged young) was assessed with a general linear model (normal distribution) in which the two potentially independent processes of gaining or losing paternity were treated as two explanatory binary factors (gaining or not, losing or not). As female fecundity is a potential source of variation in reproductive success we also tested whether clutch size was related to male traits and

female involvement in EPP with generalized linear models (Poisson distribution, and breeding date as explanatory variable).

All analyses were done in SAS 9.1 (SAS Institute, 2004). In all cases, model selection was carried out by starting from fully saturated models and removing one by one the least significant variables, starting from the highest order interactions. While performing model selection main effects were not removed before their interactions. Throughout the text means are given with 1 SE. We also present standardized effect sizes (Cohen's d) as well as their 95% confidence intervals (CI) calculated with the 'effect size calculator' by David B. Wilson (available online at http://www.campbellcollaboration.org/resources/effect_size_input.php). In order to not overestimate effect sizes in paired tests d values were computed using means and standard deviations (Dunlop et al. 1996).

RESULTS

Patterns of extra-pair paternity

31% of adults (70/213) engaged in EPP. As a consequence, 20% (106/533; 95% confidence limits: 16.5-23.3%) of the genotyped offspring, across 39% (45/113) of nests, were fathered by an extra-pair male. After exclusion of secondary females, the population level EPP rates varied slightly, the respective figures being 33% (65/198) of adults, 19% (101/517; 95% confidence limits: 16.1-22.9%) of genotyped offspring and 40% (40/99) of nests. The genetic father could be identified for 67% (71/106) of the EPY.

The probability of finding an EPY in a nest was unrelated to breeding date ($\chi^2_1 = 0.59$, $p = 0.44$, $d = 0.14$, $CI = 0.37$) or female breeding status as regards social polygamy ($\chi^2_1 = 2.00$, $p = 0.37$, $d = 0.26$, $CI = 0.37$).

Comparisons at the population level

None of the male traits analyzed had a significant influence on the probability of losing paternity (Table 2a) or gaining EPP (Table 2b). All except two of the identified males that attained EPP were at least 2 years old (yearling vs. older males: $\chi^2_1 = 5.66$, $p = 0.017$) and both young cuckolder males were also simultaneously cuckolded (Table 3). However, when the influence of breeding date ($\chi^2_1 = 12.1$, $p = 0.005$, $d = 0.74$, $CI = 0.42$) was taken into account the differences in age related to EPP success disappeared ($\chi^2_1 = 0.73$, $p = 0.39$, $d = 0.17$, $CI = 0.39$). After comparisons were restricted to older (2-5 years old) males, no differences in the probability of gaining or losing EPP were found among age classes (all comparisons $p > 0.12$, results not shown).

Gaining EPP did not influence the probability of simultaneously losing paternity (2×2 chi square table on the total values in Table 3; $\chi^2_1 = 0.98$, $p = 0.32$, $d = 0.2$, $CI = 0.39$). Thus, for identified males, gaining paternity in other nests did not imply simultaneous loss of paternity in their own nests (17 out of 25) and, similarly, loss of paternity within the own nest was not associated to gaining it in other nests (32 out of 40; Table 3). Otherwise, five males engaged in EPP with two different females whereas only three females did so with more than one extra-pair male.

Cuckolder males versus males they cuckolded

Extra-pair males had significantly larger tarsi (19.49 ± 0.10 vs. 19.17 ± 0.12 mm; $t = -2.27$, $p = 0.034$, $d = 0.63$, $CI = 0.57$), larger forehead ornaments (60.13 ± 3.17 vs. 48.81 ± 3.88 mm^2 ; $t = -2.24$, $p = 0.037$, $d = 0.71$, $CI = 0.65$) and darker plumages (92.7 ± 2.9 vs. 83.62 ± 5.1% of black feathers; $Z = 2.07$, $p = 0.038$, $d = 0.55$, $CI = 0.54$) than the social males they cuckolded (Figure 1). There were no differences between extra-pair and cuckolded males in the remainder of measured traits: wing

Table 2. Comparisons between a) males that lost and those not losing paternity and b) males that gained and those not gaining paternity in the population at large. Statistics are from a generalized linear model modelling the probability of losing (a) or gaining (b) paternity. Wing length and 1st primary with patch were standardized by age before introducing them in the models.

a)

	Estimate			χ^2_1	P	Cohen's *d*	*CI*
Forehead patch area (mm²)	-0.019	±	0.015	1.70	0.191	0.2685	0.41
Plumage blackness (%)	0.006	±	0.009	0.53	0.465	0.1498	0.4
Tarsus length (mm)	-0.145	±	0.390	0.14	0.709	0.0764	0.4
Wing length (mm)	-0.094	±	0.149	0.40	0.526	0.1301	0.41
1st primary with patch	-0.521	±	0.300	3.21	0.073	0.3824	0.42
Body weight (g)	-0.826	±	0.543	2.39	0.122	0.3213	0.41

b)

	Estimate			χ^2_1	P	Cohen's *d*	*CI*
Forehead patch area (mm²)	0.022	±	0.018	1.60	0.203	0.2604	0.4
Plumage blackness (%)	0.001	±	0.013	0.58	0.446	0.1568	0.4
Tarsus length (mm)	0.037	±	0.518	0.54	0.462	0.1504	0.4
Wing length (mm)	0.112	±	0.190	0.35	0.552	0.1216	0.4
1st primary with patch	-0.401	±	0.357	1.30	0.253	0.2408	0.41
Body weight (g)	-0.117	±	0.627	0.04	0.851	0.041	0.41

length (age corrected values: 0.19 ± 0.34 vs. -0.06 ± 0.3 mm; t = 0.54, p = 0.59, d = 0.35, CI = 0.62), white patch's first primary feather (age corrected values: -0.05 ± 0.17 vs. -0.06 ± 0.14; t = 0.03, p = 0.97, d = 0.1, CI = 0.52), body weight (12.60 ± 0.45 vs.

12.39 ± 0.42 g; t = -1.40, p = 0.18, d = 0.47, CI = 0.6) and age (2.95 ± 0.3 vs. 2.44 ± 0.24 years; Z = 0.79, p = 0.42, d = 0.41, CI = 0.57). However, the lack of age-related differences in these comparisons should be taken with caution as the identity of the cuckolder was unknown in 6 out of 11 cuckolded yearling males and hence sample sizes were small.

Cuckolder males bred, on average, 3.9 days earlier than the males they cuckolded (t = 3.06, p = 0.004). However, none of the traits predicting EPP success was related to male arrival date in earlier work (Potti and Montalvo 1991b) or to breeding date in this study (tarsus length: r = 0.037,p = 0.7, forehead patch size: r = -0.024, p = 0.8 and plumage blackness: r_s = 0.049, p = 0.62). That is, early males were not the larger or more ornamented individuals. Further, the three characters were not associated to male age and all were unrelated to each other (results not shown, all p > 0.13).

Table 3. Number of males gaining, losing paternity or simultaneously gaining and losing in the population in relation to their age.

	Yearlings	Adults	Total
Males only gaining paternity	0	17	17
Males only losing paternity	9	23	32
Males gaining and losing	2	6	8
Males neither gaining or losing	13	29	42
Total	24	74	99

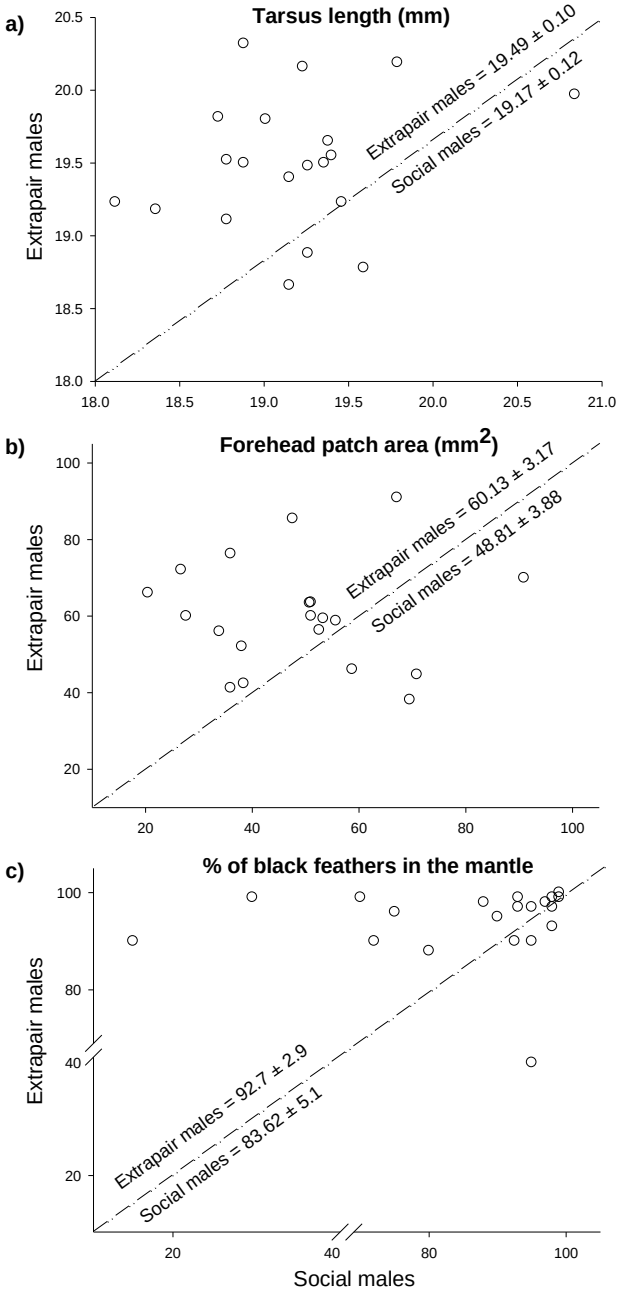

Figure 1. Comparisons of a) tarsus length, b) forehead patch size, and c) plumage blackness, in cuckolder males and the males they cuckolded. Dots above lines indicate larger values in extra-pair males than in the males they cuckolded in the analysed traits. Average (SE) population values were 19.35 (0.05) mm, 55.19 (1.39) mm^2 and 82.85 (2.3) % for tarsus length, white patch size and plumage blackness, respectively.

Influence of EPP on male reproductive output

Gaining as well as losing EPP had independent and significant influences on male reproductive output ($\chi^2_1 = 30.83$, $p < 0.001$, $d = 1.3$, $CI = 0.46$ and $\chi^2_1 = 50.51$, $p < 0.001$, $d = 1.96$, $CI = 0.54$, respectively). The interaction of both processes was marginally non significant ($\chi^2_1 = 3.44$, $p = 0.057$, $d = 0.38$, $CI = 0.4$) since the reproductive success of males involved in faithful matings was similar to that of cuckolder males simultaneously cuckolded (4.6 ± 0.2 vs. 3.8 ± 0.5; $\chi^2_1 = 1.84$, $p = 0.175$, $d = 1.7$, $CI = 0.38$). Between-groups comparisons of male reproductive success showed that cuckolder males fledged more young (7.4 ± 0.3) than males involved in faithful matings ($\chi^2_1 = 33.3$, $p < 0.001$, $d = 2.27$, $CI = 0.77$) and these, in turn, fledged more young than males exclusively losing paternity (2.4 ± 0.2; $\chi^2_1 = 41.01$, $p < 0.001$, $d = 2.22$, $CI = 0.68$).

Clutch size was unrelated to the measured male traits (all $p > 0.43$) and there was no difference in clutch size between clutches with and without EPY (5.5 ± 0.1 and 5.4 ± 0.1; $\chi^2_1 = 0.12$, $p = 0.72$, $d = 0.06$, $CI = 0.36$). The proportion of EPY per brood was 0.18 ($\pm$ 0.03) young on average, there being only 3 EPY 'pure' broods in the whole population.

DISCUSSION

We have gathered evidence for the operation of sexual selection in extra-pair mating behaviour for traits signalling male quality in the pied flycatcher. Cuckolder males were larger and displayed darker plumages and larger forehead ornaments than the males they cuckolded, results not replicated in the abundant earlier literature on this model species in sexual selection studies. Cuckolder males enjoyed increased reproductive fitness and compensated for the risk of simultaneous cuckoldry since, when this happened, their reproductive success was similar to that of males only

involved in faithful matings. This study, thus, presents additional information concerning EPP and the associated possibility of intensified sexual selection in a species for which there is contradictory information in this respect (Rätti et al. 1995; Lifjeld et al. 1997a; Dale et al. 1999; Lehtonen et al. 2009a; Moreno et al. 2010). The percentage of extra-pair young in our study (20) was higher than in most pied flycatcher populations studied to date (with reported rates ranging between 4 and 11%; Lifjeld et al. 1991; Rätti et al. 2001; Lehtonen et al. 2009a; Moreno et al. 2010), except for one Swedish locality (24%; Gelter and Tegelström 1992), which provided us with a relative large number of extra pair males, making our study more powerful as to the chance of detecting an association between male phenotype and EPP success.

As in other studies on EPP in birds (e.g. Yezerinac et al. 1995; Strohbach et al. 1998; Bitton et al. 2007; Kawano et al. 2009; Lehtonen et al. 2009a), we were unable to identify the father of a number of EPY (33%). Although in some studies this lack of resolution may be due to insufficient sampling by researchers it also may be due to other reasons. In our case, we assume that EPY with unknown fathers were sired either by males breeding outside the study area or by floaters. In general, floaters are thought to be young, low-quality or subordinate individuals, although Kempenaers et al. (2001) showed that floaters may have a significant role in EPP in tree swallows. We concur with Lehtonen et al. (2009a) in the need of more studies to understand the reproductive strategies of floaters in pied flycatchers and other avian species. In addition, although our results suggest a positive role for males with more elaborate traits in achieving EPP, caution should be taken in their interpretation since our effect size estimates had some uncertainty (broad CIs), given the low sample size in pair wise comparisons (not infrequent in behavioural ecology studies; Nakagawa, 2004; Garamszegi, 2006). In addition, our conclusions are drawn from one breeding season but EPP patterns, far from being constant, could undergo yearly variation resulting from interactions among a suite of changing ecological factors (breeding density and synchrony, operational sex ratio and/or weather conditions; Griffith et al. 2002). As

5% of nestlings were missed (due to predation or mortality prior to sampling) the estimation of EPP rate at the population level could vary slightly (± 2%, in case that all missed young were EPY or WPY).

A male's success in EPP was contingent on its phenotype. In particular, as reported in other populations (Lehtonen et al. 2009a; Moreno et al. 2010), age had a decisive influence on the probability of gaining paternity. Among those males successful in siring EPY, all except two were adult but, interestingly, the probability of gaining EPP did not vary with age among older (>1 year) males. Some authors (e.g. Wetton et al. 1995; Bouwman et al. 2007) have suggested that females engaging in EPP should prefer older males as age and, thus, long-term survival may be considered a quality indicator. In fact, male age is one of the most common factors associated to paternity in (genetically) polyandrous systems across different taxa (for references, see Kokko and Lindström, 1996; Griffith et al. 2002). However, analyses at the population level showed that breeding early was important to attain EPP for males as the number of fertile females (the necessary resource to attain EPP) may decrease with the advance of the season (Kokko et al. 2006). Hence, the high EPP success found among older males may be confounded by age-related differences in settlement and breeding phenologies. In most populations of pied flycatchers, including the southern ones, yearling males usually arrive at the breeding areas very late in the season, when most pairs are already established (Potti and Montalvo 1991b; Lundberg and Alatalo 1992). Thus, it could be argued that if finding a mate late in the season is a hard task for many yearling males, their chance to engage in EPP (if ever paired) will be even lower (Johnson et al. 2002; Chapter III). On the other hand, with almost all pair bonds already established females paired to young males should have many candidate males available to engage in episodes leading to EPP. Therefore, as our analyses accounting for breeding date highlight, the importance of male age in achieving EPP is a consequence of the typical yearling males' late phenology.

Whereas tests at the population level did not reveal any difference between cuckolder and cuckolded males, paired comparisons showed that, irrespective of their age and breeding date, extra-pair males were larger and displayed higher quality ornaments than the males they cuckolded. The discrepancies between both types of comparisons may stem from the fact that a male phenotype relative to those of his neighbours is likely to be more important for females than the male's phenotype relative to the population as a whole (Webster et al. 2001), given the rather limited female sampling of potential mates in this species (Potti and Montalvo 1991b; Dale and Slagsvold 1996).

In avian species, male variation in the ability to attain EPP or, alternatively, avoid loss of paternity have been commonly related to size (Weatherhead and Boag, 1995; Neto et al. 2010) and quality of plumage ornaments (e.g. Kempenaers et al. 1997; Cordero et al. 1999; Bitton et al. 2007). Examples of multiple traits being sexually selected are also frequent, though their role in determining male reproductive success remains unclear (reviewed in Candolin 2003). From an adaptive point of view (Møller and Pomiankowski 1993), multiple traits may signal multiple qualities (Jawor et al. 2004; Van Doorn and Weissing 2004;), be redundant signals of the same aspect of quality allowing more accurate individual assessments (Zuk et al.1992; Candolin and Voigt 2001) or not indicate male quality but facilitate detection or signal reception (Pomiankowski and Iwasa 1993; Iwasa and Pomi ankowski 1994). Previous work with European flycatchers indicates that all male traits conferring success in our study have significant heritability in several populations (Alatalo and Lundberg 1986; Potti and Merino 1994; Qvarnström 1999; Lehtonen et al. 2009b; Potti and Canal 2011) and are likely honest signals of male quality (e.g. Potti and Montalvo 1991b; Slagsvold and Lifjeld 1992; Sætre et al. 1994; Gustafsson et al. 1995; Sheldon et al. 1997; Sirkiä and Laaksonen 2009). Further, all favoured traits were uncorrelated, suggesting that each one may signal a different aspect of quality, as in zebra finches (*Taeniopygia guttata*; Birkhead et al. 1998) or great tits (*Parus major*; Rivera Gutierrez et al. 2010; see also

references in Candolin 2003). In pied flycatchers, a large size, as scored by tarsus length, may be beneficial in intrasexual competition (Sirkiä and Laaksonen 2009) and is indirectly related to fledgling survival (through its relationship with body condition; Alatalo et al. 1990). Plumage colour is also related to individual quality in this species, as darker males are the first to establish breeding territories on the arrival from spring migration (Lundberg and Alatalo 1992), have larger song repertories (Lampe and Espmark 1994) and feed their chicks at higher rates than browner males (Sætre et al. 1995). The white forehead patch functions as a badge of status in black-and-white European flycatchers (Qvarnström 1997; Sanz 2001), with large ornamented males enjoying competitive advantages both in male conflicts over nest sites and in acquiring females more quickly (Potti and Montalvo 1991b; Pärt and Qvarnström 1997). Moreover, in collared flycatchers size of the forehead patch is related to male lifetime reproductive success and the likelihood of becoming polygynous and losing paternity (Gustafsson et al. 1995; Sheldon et al. 1997).

Discrepancies across same species' populations in traits involved in extra- pair paternity contexts are not rare in the literature (e.g. great tit; Strohbach et al. 1998; Kawano et al. 2009; blue tits *Cyanistes caeruleus*; Kempenaers et al. 1992, 1997; Charmantier et al. 2004; red-winged blackbirds *Agelaius phoeniceus*; Weatherhead and Boag 1995; Wesneat 2006; reviewed in Akçay and Roughgarden 2007) and the pied flycatcher is not an exception as no trait except age has been found to be associated to success in EPP in most previous studies of the species (Rätti et al. 1995; Slagsvold et al. 2001; Moreno et al. 2010; but see Lehtonen et al. 2009a). Differences among populations may be due to past selection on those traits being nowadays weak or non-existent in some of them (Dale et al. 1999), which would influence what traits are currently being selected in (extra- and within-pair) mating behaviour (Endler and Houde 1995; Westneat et al. 2006; Dunn et al. 2008). Additionally, breeding synchrony and density may determine how male and female behaviour interact to give the different EPP rates and patterns seen across populations (Griffith et al. 2002;

Westneat and Stewart 2003). Many studies concerning EPP, especially the early ones, may have failed in the detection of phenotypic traits involved in EPP contexts due to low sample sizes (Lubjuhn et al. 2007; see also appendix 2 in Griffith et al. 2002).

Extra-pair paternity may strengthen sexual selection when paternity gains are related to particular male traits and are not counteracted by similar losses of WPY (Webster et al. 1995) as, in this case, there will be little or no influence of EPP on the total male reproductive output (Freeman-Galllant et al. 2005). In fact, some studies suggest that EPP does not boost male fitness in monogamous species as much (e.g. Dunn et al. 2001; Webster et al. 2001; Whittingham and Dunn 2005) as initially assumed (Birkhead and Møller 1992; Møller and Ninni 1998). In our population, siring additional young increased reproductive success because gaining EPP did not imply concurrent loss of paternity, i.e. most males gaining paternity did not lose it in their own nests. Comparisons among males revealed that cuckolders sired 2 more young, on average, than males engaging in faithful matings (and 5 more young than cuckolded males). Our results regarding male fitness are similar to those recently found in mountain bluebirds (*Sialia currucoides*; Balenger et al. 2008) but differ from those in species, as the yellow warbler (*Dendroica petechia*), in which EPP may dramatically increase male fitness (Yezerinac et al. 1995), likely because most males engaged only in one EPP mating and pure EPY broods were infrequent. Interestingly, from a point of view of loss of paternity, the rarity of pure EPY broods entailed that success in attaining EPP compensated for the risks of simultaneous cuckoldry because, when that happened, the males' reproductive output was similar to that of males only engaged in faithful matings.

It is tempting to suggest, on the basis of our results, that females may be able to assess and compare, based on the males' phenotypes, the quality of their social and EPP mates (Jennions and Petrie 2000). As a consequence, females paired with high quality males may be less prone to promiscuity whereas those paired with low quality males may actively solicit extra-pair copulations in order to gain some kind of benefit

(Kempenaers et al. 1992). An alternative explanation is that cuckolder, high quality males may either invest more in pursuing extra-pair copulations, or be more capable of attaining these (Alatalo et al. 1987; Weatherhead and Boag 1995; Dunn and Cockburn 1999; Bitton, 2007) or in enforcing copulations from reluctant females (Arnqvist and Kirkpatrick 2005). Most likely, however, our results arise from the interactions of females with their social and EPP mates being contingent on a set of ecological factors constraining EPP opportunities (Westneat and Stewart 2003; van Dongen and Mulder 2009). That cuckolder males are not completely safe from cuckoldry may be explained by the fact that females paired with high quality males may have male neighbours of even higher quality (Akçay and Roughgarden 2007).

To conclude, we have identified some predictors of male success in extra-pair paternity and shown the importance of this behaviour in boosting male reproductive fitness. These findings suggest that EPP may be contributing to the evolution of the selected male traits in this population. Our results contrast with those of previous work in pied flycatchers and highlight the divergence on traits related to EPP success among populations. It remains a task for the future to experimentally ascertain to what extent these results are a consequence of female preference for particular male characters or due to a higher ability of large males displaying more elaborate traits to secure genetic polygamy.

CAPITULO III

Multiple mating opportunities boost protandry in a pied flycatcher population

Macho y hembra de papamoscas construyendo el nido. Foto: Miklos Laczi

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http://link.springer.com/article/10.1007%2Fs00265-011-1253-8?LI=true

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ABSTRACT

Protandry, the earlier arrival of males than females to breeding areas, is widespread in birds, but its underlying mechanisms are far from well understood. The two, not mutually exclusive most highly supported hypotheses to explain avian protandry postulate that it has evolved from intrasexual male competition to acquire the best territories ("rank advantage" hypothesis) and/or to maximize the number of mates ("mate opportunity" hypothesis). We studied for two consecutive years the relative importance of both hypotheses in a population of pied flycatchers (*Ficedula hypoleuca*), a territorial songbird with a mixed mating strategy. We measured territory quality using a long-term dataset on nest occupation and breeding output, and we used molecular techniques to assess male fitness across the range of social and genetic mating options. Territory quality was unrelated to breeding date and had no influence on extra-pair paternity or social polygynous events. However, males breeding early increased their chances of becoming socially polygynous and/or of attaining extra-pair paternity and, as a consequence, increased their total reproductive success. These results support the "mate opportunity" hypothesis, suggesting that sexual selection is the main mechanism driving protandry in this population.

Keywords: Extra-pair paternity, Ficedula hypoleuca, mate opportunity hypothesis, protandry, rank advantage hypothesis, social polygyny

INTRODUCTION

Males and females emerge asynchronously, or arrive at different times at the breeding areas, in many taxa including insects, amphibians, birds, fishes and mammals (reviewed by Morbey and Ydenberg 2001). Protandry, the earlier arrival/emergence of males than females, is the most widespread pattern (e.g. Morbey and Ydenberg 2001; Kokko et al. 2006; but see Reynolds et al. 1986). Earlier males often show higher reproductive success (Thornhill and Alcock 1983; Newton 2008), especially when female fecundity decreases with time (Kleckner et al. 1995; Carvalho et al. 1998), but the mechanisms underlying protandry are not well understood.

Several hypotheses aim to explain whether selection acts directly or indirectly on the difference between male and female timing of arrival (Morbey and Ydenberg 2001). Given the diversity of the mating systems wherein protandry occurs, the different hypotheses apply to different groups. In insects, for instance, protandry may be a by-product of selection for larger (implying longer developmental time) females than males when female's reproductive capacity increases with size (the "constraint hypothesis"; e.g. Wiklund and Solbreck 1982; Thornhill and Alcock 1983). In some lizards, however, males are incapable to reproduce immediately after emergence, and selection may act directly on the female's timing of emergence by delaying it to reduce the odds of mating with infertile individuals (the "waiting cost hypothesis"; e.g. Olsson and Madsen 1996). In birds, where protandry is common (Rubolini et al. 2004; Coppack et al. 2006; Newton 2008) the two, not mutually exclusive, most strongly supported hypotheses explaining protandry are the "rank advantage" (Ketterson and Nolan 1976; Kokko 1999) and the "mate opportunity" hypothesis (originally conceived in butterflies; Wiklund and Fagerström 1977). The "rank advantage" hypothesis postulates that competition for gaining the best territories is the selective force driving the sex differences in arrival schedules (Ketterson and Nolan 1976). Accordingly, an enhanced breeding success for early arriving males has been

associated with acquisition of the best territories (Alatalo et al. 1986; Forstmeier 2002), and the sex defending a crucial resource for breeding (e.g. a territory or a nest site) usually arrives first (e.g. Myers 1981; Alatalo et al. 1986; Hasselquist 1998). Conversely, under the "mate opportunity" hypothesis, selection will favor protandry if males maximize their mating opportunities through an early arrival (Lozano et al. 1996; Langefors et al. 1998). This is especially important for species with a mixed mating strategy wherein early breeding may allow the consecution of additional matings via social polygyny and/or extra-pair paternity (EPP hereafter; Reudink et al. 2009). In support of this hypothesis, the chances of multiple mating either in social polygyny (Hasselquist 1998) or via EPP (Langefors et al. 1998; Coppack et al. 2006) have been shown to increase with early male arrival. In fact, the commonness of EPP (Griffith et al. 2002; Westneat and Stewart 2003) has given impetus to the "mate opportunity" hypothesis as the main mechanism underlying the evolution of protandry at both the within- and the between-species levels (e.g. Langefors et al. 1998; Rubolini et al. 2004; Coppack et al. 2006; Kokko et al. 2006; Møller et al. 2009; Reudink et al. 2009; but see Saino et al. 2010). Given that male (more than female) fitness is tightly correlated to the number of matings they achieve (Andersson 1994), males arriving simultaneously or later than females will lose as many mating opportunities as the number of females that were receptive before male arrival (Kokko et al. 2006).

As pointed out by Morbey and Ydenberg (2001), studies should simultaneously consider the significance of the different selective pressures, given that hypotheses of protandry are not mutually exclusive. However, to our knowledge, no study has simultaneously analyzed, in a single species, the different factors underlying the two main hypotheses related to protandry in birds, i.e. the "rank advantage" and "mate opportunity" hypotheses, accounting for both EPP and/or social polygyny. Here, we did so studying a population of pied flycatchers (*Ficedula hypoleuca*), an interesting species in this regard because most males arrive at the breeding areas before females (Potti and Montalvo 1991b), and establish a territory around a nest site

and subsequently try to attract a female, thus allowing for testing of the relevance of territory quality (Lundberg and Alatalo 1992). The mating system is mainly monogamous, but 3-25% of the males acquire a second mate (secondary female, hereafter), becoming socially polygynous (reviewed in Lundberg and Alatalo 1992). Moreover, genetic polygyny is common, with percentages of extra-pair young (EPY hereafter) ranging from 4 to 24% (Table 2 in Rätti et al. 2001; see Chapter II and references therein). Here, we studied the relative importance of the main mechanisms proposed to promote avian protandry (territory quality versus mating opportunities) by using molecular techniques to track the fitness of males through EPP and social polygyny in combination with an ongoing long-term study to estimate territory quality.

MATERIAL AND METHODS

Field work

The study was carried out during the breeding seasons of 2005 and 2006 as part of a long-term study of pied flycatchers in central Spain (Potti y Montalvo 1991a,b; Potti y Canal 2011). The study area consists of two plots (located in an oak wood and a pinewood) 1.3 km apart, including 236 nest-boxes which positions have remained stable since 1995. Universal Transverse Mercator (UTM) coordinates of all nests were GPS (Global Positioning System) referenced and distances among them calculated with Arcview (ESRI 2000is™). The average (SD) minimum distance between occupied nest-boxes was 30 (14.1) m.

Field protocols have been described in the Chapter II (see also Potti and Montalvo 1991a,b). Briefly, all nests were regularly checked (every 3 days before laying started and on a daily basis around hatching) to ascertain laying date, clutch size, hatching date and number of fledglings. Parent birds were captured with a nest-box trap while they were feeding 8-day-old nestlings. Birds were weighed (with a spring balance, to the nearest 0.1 g) and measured for tarsus length (with callipers, to the

nearest 0.01 mm), height and width of the forehead patch (to the nearest 0.01 mm) and wing length (with a ruler, to the nearest 0.5 mm). The area of the forehead patch was calculated as patch height × width. Fledglings were measured and weighed at 13 days of age. Blood samples were taken from all individuals by puncturing the brachial vein and stored in ethanol.

Molecular methods

Our sample size for parentage analyses was 1,568 individuals: 531 chicks and 212 adults (113 females and 99 males) from 113 nests in 2005 and 595 chicks and 229 adults (120 females and 109 males) from 120 nests in 2006. Within-year discrepancies in male and female numbers are due to bigamous pairings. Additional data from females lacking male assistance involving 8/21 and 8/22 females/chicks in 2005 and 2006, respectively, were excluded from analyses (see below). Individuals from 2005 were genotyped at seven polymorphic microsatellite loci (fhu1 and fhu2 (Ellegren 1992), fhu3 and fhu4 (Primmer et al. 1996) and Fhy6-126, Fhy1-25, Fhy3-60 (Chapter I)). To further increase reliability in the assignment of genetic fathers we genotyped all individuals from nests containing young having mismatches with their putative father (see below) with three additional primers (fhy444, fhy466 and fhy310; Leder et al., 2008). Individuals from 2006 were genotyped at fifteen microsatellite isolated (f3-60, f1-25 (Canal et al. 2009) and fhy 216, fhy 237, fhy 301, fhy 304, fhy 310, fhy 329, fhy 339, fhy 356, fhy 361, fhy 401, fhy 444, fhy 466 and fhy 236 (Leder et al. 2008); see Chapter V). We identified a given male as an extra-pair sire when an EPY had no or one mismatch and a high likelihood score with him. Paternity assignments were based on a 95% confidence level (see Chapter II for further details on paternity analysis).

Mating opportunities and breeding phenology

Egg laying dates (scored as days after 1 May) were used as a proxy for arrival dates. We are confident in this approach because we have previously shown a strong correlation between both variables in the study population (Potti and Montalvo 1991b), a fact also reported in other populations of pied flycatchers (Alatalo et al. 1986; Lundberg and Alatalo 1992) and in many other avian species (e.g. Møller 1994; Bêty et al. 2003; Cooper et al. 2009).

When working simultaneously with EPP and social polygyny, some considerations were taken into account since the inclusion in the analyses of different types of individuals such as secondary females with or without male assistance and/or those engaging or not in EPP may be problematic. First, the secondary status may affect paternity of the offspring if males spend less time potentially guarding females during their fertile period (Lundberg and Alatalo 1992). However, data from secondary broods with male assistance were considered in the analyses concerning EPP since our aim here is to study the adaptive mechanism (s) promoting protandry and not those promoting EPP (i.e. we aimed to assess male fitness accrued from EPP and not the reasons for female promiscuous behavior). Second, data from females lacking male assistance were excluded from analyses as they could, in fact, be secondary females or either have been deserted by their mates or widowed after pairing. To confirm that those cases did not bias our conclusions in polygynous contexts, we made the analyses including and excluding data from nests lacking male assistance and results remained unchanged (data not shown).

Temporal patterns of EPP and social polygyny

The probabilities of males and females being involved in EPP (coded as 0/1) during the breeding season were modeled in each year with generalized linear models

(binomial distribution) and laying dates as explanatory variables. Likewise, the probabilities of a male becoming socially polygynous or a female becoming secondary were modeled in each year.

The influence of laying date on male fitness was tested with general or generalized linear models. In these analyses, the reproductive success of males (number of fledglings sired) was divided into several components: fitness attributable to the social pair (once those fledglings lost by EPP were deducted from their own nests (normal distribution)), that due to additional matings (Poisson distribution), and overall realized reproductive success (normal distribution).

Territory quality

The long-term quality of territories (nest-boxes; n = 236) was calculated using information from a period of 16 years (1995-2010). To this end, we computed an index of nest occupancy (following Sergio and Newton 2003; see also Askenmo 1984) as the proportion of years a nest-box was occupied by pied flycatchers in relation to those it was available (i.e. not occupied by other species). The index thus shows the preference of the species for each nest-box since, on average (range), 34 (5-56) % of the nest-boxes were not occupied but available to the flycatchers each year and the proportion of nest-boxes used by other species is relatively low (on average, 12 (3-31) %). We also computed two additional indices indicative of territory quality based on the mean numbers of nestlings fledged and of those recruited from each nest-box in the following years, thus summarizing all the factors potentially shaping the breeding success in a given territory, and the survival expectancies of the chicks reared there. As the three indices were intercorrelated but not fully redundant (occupancy-number of fledglings: $r_s = 0.15$, $P = 0.017$, occupancy-number of recruits: $r_s = 0.17$, $P = 0.007$, number of fledglings-recruits: $r_s = 0.32$, $P < 0.001$), we made a Principal Component Analysis to summarize overall variation in territory quality. PC1 explained 48.2% of

the total variance in territory quality with similar and positive contribution of each index (factor loadings: occupation rate 0.56, number of fledglings 0.75, number of recruits 0.76) and its scores were used as an index of territory quality in further analyses (tests done with each index separately gave similar results, results not shown).

We used a general linear model to test whether territory quality was related to breeding date or annual reproductive success (numbers of fledglings and recruits). A generalized linear model was run to see whether males that attained EPP (binomial distribution) occupied the best territories wherein male identity was introduced as a random factor (as some males bred in both years). Wilcoxon tests were used to see if promiscuous females engaged in EPP with males established in better territories than those of their social males. Likewise, in contexts of social polygyny, the relationship between female mating status (primary, secondary or monogamous) and the quality of her (social male's) territory was modeled with a generalized linear model where female status was introduced as a multinomial dependent variable, territory quality as an explanatory variable and female identity as a random factor. Primary and secondary territories of polygynous males were also compared with pairwise tests.

An association between territory and male quality could be an important and potential confounding source of variation concerning conclusions on the ranking advantage hypothesis of protandry. To explore this possibility, we calculated the average size of male forehead patches and tarsus lengths (two traits positively related to success in intrasexual competition for territories; see Lundberg and Alatalo 1992; Sanz 2001) for males occupying a given nest-box, and related them to the scores of territory quality with general linear models. In addition, we related long-term territory quality with male traits in the two study years separately.

Statistical analyses were made in SAS 9.1 (SAS Institute 2004) and Statistica 7 (StatSoft, Inc. 2004).

RESULTS

Patterns of EPP and social polygyny

In 2005, 40% (45/113) of the nests and 33% (70/212) of the adults were involved in EPP episodes with 20% (106/531) of the offspring being EPY. Respective figures in 2006 were a bit lower: 27% (32/120), 21% (48/229) and 11% (68/595), respectively. Excluding secondary females, these rates were: 40% (40/99), 33% (65/198) and 19% (101/517) in 2005 and 28% (30/108), 21% (46/216) and 12% (65/546) in 2006. Regarding social polygyny, we were able to identify the bigamous male parent in 14 and 11 nests in 2005 and 2006, respectively.

In 2005, the probability of a male engaging in EPP was highest at the beginning of the breeding season, decreasing afterwards ($\chi^2_1 = 8.2$, $P = 0.004$). The same trend was observed in 2006, but the relationship was not statistically significant ($\chi^2_1 = 0.18$, $P = 0.67$; Fig. 1). The probability of females engaging in EPP was unrelated to breeding date in both years ($\chi^2_1 = 0.01$, $P = 0.98$ and $\chi^2_1 = 1.26$, $P = 0.26$, in 2005 and 2006, respectively; Fig. 2).

In both years, the probability of a male becoming socially polygynous decreased as the season advanced ($\chi^2_1 = 11.46$, $P < 0.001$ and $\chi^2_1 = 4.15$, $P = 0.041$, in 2005 and 2006, respectively; Fig. 1). In contrast, the probability of becoming a secondary female increased throughout the season ($\chi^2_1 = 8.70$, $P = 0.003$ and $\chi^2_1 = 3.79$, $P = 0.05$; Fig. 2).

Male fitness decreased with laying date in both years ($\chi^2_1 = 19.07$, $P < 0.001$ and $\chi^2_1 = 9.75$, $P = 0.002$, in 2005 and 2006, respectively). This was explained in part by the number of young attributable to the social pair ($\chi^2_1 = 6.89$, $P = 0.008$ and $\chi^2_1 = 9.99$, $P = 0.002$), but particularly because early breeding males increased their fitness by siring young through EPP and/or by becoming polygynous in other nests ($\chi^2_1 = 13.72$, $P < 0.001$ and $\chi^2_1 = 3.3$, $P = 0.069$; Fig. 3).

Figure 1. Male probability of attaining extra-pair paternity (top) or becoming socially polygynous (middle), and territory quality achieved (bottom) according to the laying date (as days after 1 May) of thei social pair.

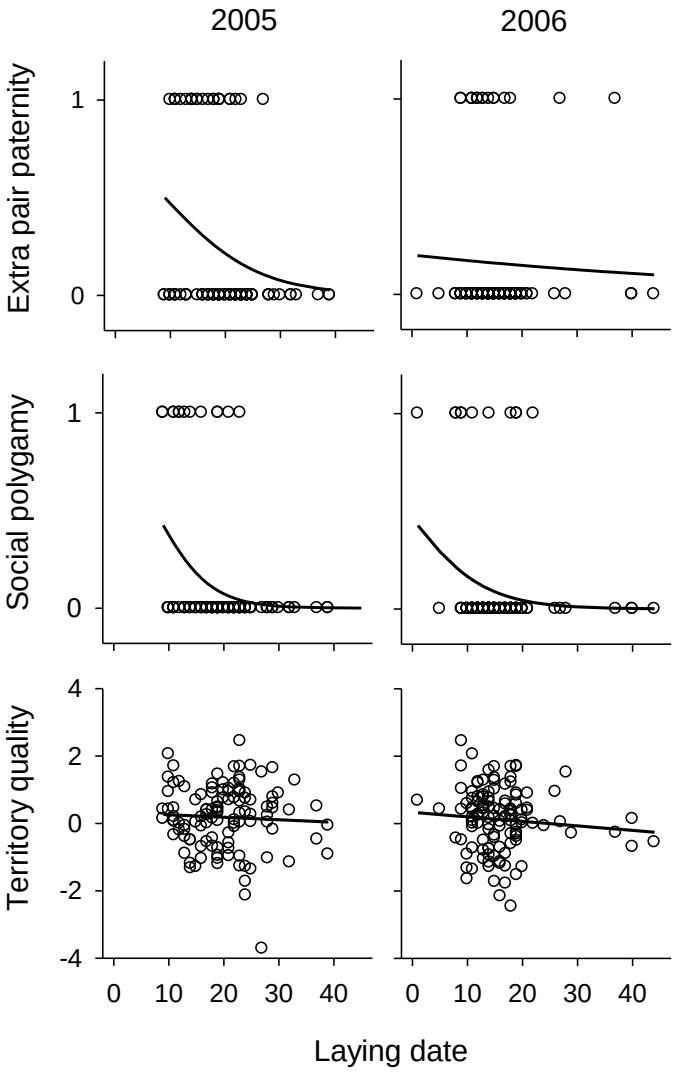
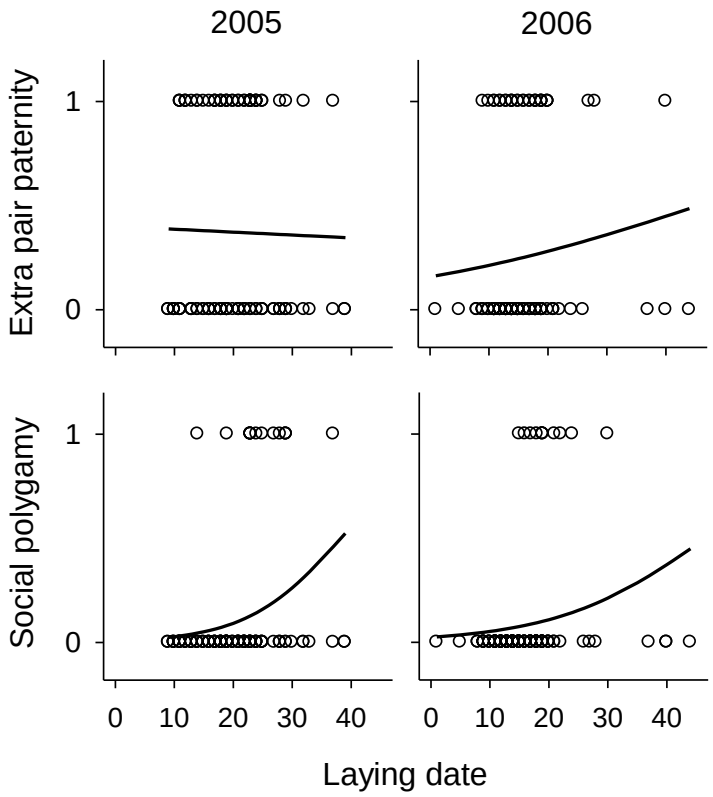


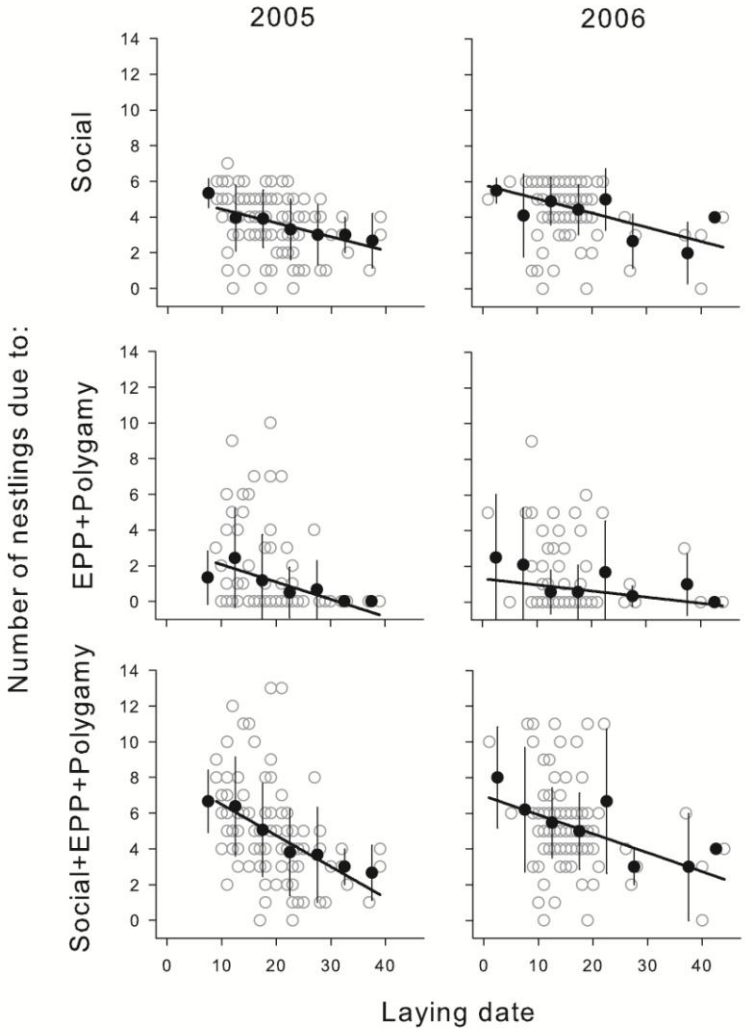
Figure 2. Female probability of engaging in extra-pair paternity (top) or becoming secondary (bottom) in relation to laying date (as days after 1 May).



Extra-pair mating, social polygyny and territory quality

Long-term territory quality was not associated with average size (tarsus length; χ^2_1 = 1.57, P = 0.21) or forehead patch size (χ^2_1 = 0.01, P = 0.97) of the males occupying the nest-boxes. The same was true when limiting the analysis to 2005 and 2006 (tarsus length: χ^2_1 =1.74, P = 0.18 and χ^2_1 = 0.01, P = 0.93; forehead patch size: χ^2_1 = 1.22, P = 0.26 and χ^2_1 = 0.14, P = 0.7). These results suggest that an association between male quality and prime territory sites (see Alatalo et al. 1986) is not likely to be biasing

Figure 3. Relationship between male fitness and the laying date (as days after 1 May) of their social pair. Male fitness is divided into two components: fitness attributable to the social pair (top), and fitness due to additional matings (middle). Overall reproductive output is also shown (bottom). Grey empty dots show raw data (one dot for each male); black dots show the mean (SD) in bins of 5 days (i.e., laying date 1–5, 6–10, 11–15, etc.), showing that the data followed the overall linear trend along the entire laying date range.



our results herein. Territory quality was independent of laying date as early breeders did not occupy better territories (all nests $\chi^2_1 = 0.03$, P = 0.86 and $\chi^2_1 = 0.99$, P = 0.31; after removing secondary nests: $\chi^2_1 = 0.15$, P = 0.7, and $\chi^2_1 = 1.14$, P = 0.28, in 2005 and 2006, respectively, Fig. 1). Territory quality did not influence EPP or social polygyny events, as extra-pair males did not occupy better territories than males not involved in EPP ($\chi^2_1 = 0.07$, P = 0.79), neither did females engage in EPP with males holding better territories than those of their social mates (Z = 0.24, P = 0.8). Also, there were no differences in the quality of the territories between monogamous, primary or secondary females ($\chi^2_1 = 0.29$, P = 0.59) and the primary and secondary territories of polygynous males were of similar quality (Z = 0.29, P = 0.76). Long-term territory quality was unrelated to the annual production of fledglings (2005: $\chi^2_1 = 0.24$, P = 0.62; 2006: $\chi^2_1 = 1.3$, P = 0.25) and recruits (2005: $\chi^2_1 = 0.09$, P = 0.76; 2006: $\chi^2_1 = 0.01$, P = 0.91).

DISCUSSION

Breeding early was advantageous for males as their chances to become polygynous and engaging in extra-pair matings declined through the season, even though for the latter there was annual variation in the significance of arriving early to the breeding grounds. Although males increased their reproductive output by breeding early, the increase was higher for males siring young in several nests. Conversely, females were not constrained to maximize their EPP opportunities through early breeding as their likelihood to engage in EPP was unrelated to date, though their chances of becoming secondary increased throughout the season. Remarkably, territory quality was not related to breeding date, nor was it influenced by EPP or polygynous events; EPP males did not occupy better territories than the males they cuckolded and the same was true for primary and secondary territories of polygynous males. Since by breeding

early males improved their prospects for multiple matings and, in turn, their fitness, it follows that sexual selection may be underlying protandry in this population.

The exact moment when extra-pair fertilizations occur may seem uncertain since female birds are known to store sperm from a few days to several weeks (Birkhead and Møller 1992; Birkhead 1998). However, due to sperm competition, early extra-pair copulations (EPC) have reduced chances of success since any subsequent copulation with the social male seems to decrease the fertilization success from prior inseminations via last-male sperm precedence (Birkhead and Møller 1992; Birkhead 1998; Michl et al. 2002). In fact, the highest rate of pair copulations and fertilizations in pied flycatchers occurs between days -2 and -1 (Lifjeld et al. 1997b) whereas experimental work with the sister species (the collared flycatcher, Ficedula albicollis; Michl et al. 2002), suggests that females may be selectively timing EPC to the period comprised between days 0 and +1 (Michl et al. 2002). For these reasons, we consider any potential effect of that uncertainty on our conclusions small.

A plethora of studies has shown that an early reproduction is one of the main determinants of breeding success in seasonally breeding taxa (e.g. mosquitoes, Kleckner et al. 1995; butterflies, Carvalho et al. 1998; birds, Table 14.2 in Newton 2008). However, an early phenology could impose costs due to adverse environmental conditions in the breeding areas at the beginning of the season (e.g. Morton and Sherman 1978; Crecco and Savoy 1985; Newton 2008). In birds, early arrival has been suggested to provide reliable information of male quality for females because only males in good condition can afford to arrive early (e.g. Arvidsson and Neergaard 1991; Lozano 1994; Møller 1994; Kissner et al. 2003, Møller et al. 2003, Smith and Moore 2005) due, for instance, to the risk of mortality by harsh environmental conditions (Brown and Brown 2000; Møller 2004; see also Table 4 in Newton 2006). Males, therefore, face a trade-off between the advantages and risks associated with an early arrival so that protandry should only appear when benefits for early arriving males outweigh costs derived from natural selection (Kokko 1999; Spottiswoode et al. 2006).

Both theoretical and empirical studies imply that competition for the best territories and increased mating success EPP strengthens selection for an early arrival in males as compared with females (Thornhill and Alcock 1983; Morbey and Ydenberg 2001; Kokko et al. 2006 and references therein). In some wasps and butterflies, protandry via mate opportunity is favored when females mate once, when most eggs are laid after the first mating or when sperm precedence of the first male occurs (Wiklund and Fagerström 1977; Thornhill and Alcock 1983; Hastings 1989). Likewise, in some fishes and newts first males increase their chances of multiple matings and/or of siring more offspring (Morbey 2000; Tennessen and Zamudio 2003). In territorial birds, however, the acquisition of the best territories or resources selects for the earlier arrival of the territorial sex (Ketterson and Nolan 1976). In our population, early breeders enjoyed greater chances of additional matings than late breeders. By contrast, territory quality was unrelated to breeding date. Further, females did not attain EPP with males holding better territories nor did secondary females occupy worse territories than their male's primary territory. Similarly, at the population level extra-pair males did not occupy better territories than males not engaging in EPP, nor did territory quality vary across the range of social mating types (monogamous, primary or secondary pairings). Thus, habitat features seem not to be heterogeneous enough in our study area to promote protandry through competition for the best territories. Alternatively, if territory quality fluctuates widely from year to year, long-term quality measures (of occupancy and/or productivity) may not reflect territory quality in a particular year (Sergio and Newton 2003) as suggested by the lack of correlation between our long-term quality indexes and yearly reproductive success in the territories. Experimental settings (e.g. Alatalo et al. 1986; Lifjeld and Slagsvold 1988; Sirkiä and Laaksonen 2009) will surely provide further insights into female choice of male(s) characteristics and territory quality and their consequences for multiple mating. Recently, Kokko et al. (2006) have shown that the rank advantage hypothesis per se may fail to explain protandry in migrant birds since females are also

expected to advance their arrival date (even more than males) when arriving late affects their fitness (e.g. by occupying poor territories). In fact, empirical work at both within- and between-species levels has confirmed that competition for mates rather than territories positively influences protandry (Rubolini et al. 2004; Coppack et al. 2006; Møller et al. 2003, 2009). In accordance with these studies, the prospects of additional paternity seem to be the main factor promoting selection for an early social mate acquisition and thus an early male arrival in our population. Additionally, a male-biased adult sex ratio could be operating together with social polygyny and EPP in strengthening selection for protandry by accentuating within-sex competition for mates (Kokko et al. 2006). However, as in other previous studies (e.g. Rubolini et al. 2004; Coppack et al. 2006; Saino et al. 2010), this association was not studied here due to the difficulty in obtaining reliable estimates of tertiary sex ratios in wild populations.

At least two factors, i.e. availability of fertile females and scarcity of competitors for mates, may influence multiple matings opportunities for early breeding males (Thornhill and Alcock 1983; Hastings 1989; Holzapfel and Bradshaw 2002). In our pied flycatcher population, most females were likely either arriving or still fertile when early males had already paired, which should increase the males' chances of multiple matings. Further, as few competitors for additional (genetic/social) matings would be present in the early stages of the breeding season this would increase the chances of gaining paternity while at the same time reducing those of cuckoldry (Birkhead and Møller, 1998; Fishman et al. 2003). From the female point of view, mating with early (i.e. high quality) males in extra-pair contexts could provide some type of direct/indirect benefits (e.g. Møller 1994; Lozano et al. 1996; Møller et al. 2003; Smith and Moore 2005). In fact, a number of studies, including one in this population (Chapter II), show that EPP success covaries with male traits signaling quality (plumage ornamentation or song repertory; e.g. Weatherhead and Boag 1995; Kempenaers et al. 1997; Cordero et al. 1999; Bitton et al. 2007; Neto et al. 2010, see also Appendix 2 in Griffith et al. 2002). On the other hand, females mated

with (early) polygynous males could benefit in future generations by the enhanced fitness of their offspring (e.g. by inheriting their fathers attractiveness; Weatherhead and Robertson 1979) despite suffering direct costs in their current reproductive success. Empirical studies dealing with the latter prediction have nonetheless reported contrasting results (Huk and Winkel 2008, see also Ligon 1999).

Phenological trends of genetic polygyny varied slightly between both study years. In 2006, a marked advancement (6 days) in the mean population breeding date with respect to the historical population mean ($t = 9.42$, $P < 0.001$) caused a decrease in the effective time to attain EPP. By contrast, the opportunities of becoming socially polygynous were not apparently affected by such advancement, likely because secondary females usually breed late in the season (Lundberg and Alatalo 1992; Fig. 2), contrary to the case of females engaging in EPP (Fig. 2). Since the variance in the number of mates strongly affects male fitness (Andersson 1994; Webster et al. 2007), our study highlights the adaptive importance of an early breeding (and hence, arrival) for males. Males should settle especially early in years wherein females rapidly become a scarce resource since a delay in their arrival may generate great loss of fitness opportunities (Kokko et al. 2006). The optimal arrival moment for males should depend on the interaction between individual phenotype (its physical condition) and environment (changing ecological factors) since mortality rates are high early in the season and the onset of breeding likely matches food availability (Brown and Brown 2000; Jonzén et al. 2007). In contrast, males arriving simultaneously or later than females will lose mating prospects at a rate proportional to the number of females becoming infertile in the population each day, i.e. late arrived males will not be able to mate with those females already incubating or rearing their chicks (Kokko 1999; Kokko et al. 2006).

To conclude, we found little support for territory quality favoring the evolution of patterns in breeding phenology or in (social and/or genetic) polygyny in this pied flycatcher population (i.e. the "rank advantage" hypothesis). However, our

data provide supporting evidence for an increase in reproductive output for the earliest arriving males through higher success in both socially and genetically polygynous settings (i.e. "mating opportunity" hypothesis). Since EPP and social polygyny confer great advantages in male reproductive success, sexual selection may be underlying the different schedules in the arrival dates of males and females in this population.

CAPITULO IV

Male decisions or female accessibility? Spatiotemporal patterns of extra pair paternity in a songbird

Hembra y macho de papamoscas apoyados en la caja nido.
Fotos: Carlos Camacho y David Canal

Canal D, Jovani, R, Potti, J. Male decisions or female accessibility? Spatiotemporal patterns of extra pair paternity in a songbird. Behav Ecol. 23: 1146-1153.
http://beheco.oxfordjournals.org/content/23/5/1146.abstract

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ABSTRACT

Extra pair paternity (EPP) is widespread in birds, but its high variability across years, populations and species is to a great extent unresolved. Here we explored during two breeding seasons population and individual accessibility to fertile females at different spatiotemporal scales in a population of pied flycatchers (Ficedula hypoleuca) to understand whether individual patterns of EPP were due to adaptive individual behavior or to ecological constraints. Our aim was to comprehend variation in EPP population patterns through the understanding of individual behavior. At the population level, EPP probability decayed with distance between nests. At the individual level, however, males engaged in EPP with distant (up to 390 m) females despite the fact that there were often fertile females in closer territories. EPP cases occurred mostly during egg laying and the incubation of the extra pair male's social female despite that other neighboring females were fertile before and after these periods. Results suggest a male strategy to maximize reproductive output by guarding their social females during their peak of fertility, seeking EPP afterwards and investing in parental duties once their social nestlings hatch. This may explain why EPP rate was higher in the year with lower breeding synchrony, because this allowed early-breeding males to have more EPP opportunities after their social mate laying onset. This study highlights the necessity of considering the social contexts of individuals at the spatiotemporal scales at which EPP takes place to understand variation in EPP patterns at the individual and population levels.

Keywords: breeding phenology, Ficedula hypoleuca, genetic polygamy, pied flycatcher, population density, synchrony.

INTRODUCTION

Multiple paternity is common in animals (e.g. fish, Sefc et al. 2008; mammals, Cohas and Allainé 2009; birds, Griffith et al. 2002). Although monogamy is the commonest breeding system in birds (Lack 1968; Birkhead and Møller 1992; Bennett and Owens 2002), many studies in socially monogamous species in the last two decades have shown that genetic polygamy (i.e. extra pair paternity; EPP) is widespread (Griffith et al. 2002). Studies on EPP have typically focused on the adaptiveness of this behavior for both males and females under cost-benefit frameworks (reviewed in Jennions and Petrie 2000; Arnqvist and Kirkpatrick 2005; Akçay and Roughgarden 2007; Mays et al. 2008). However, the large variation in EPP rates among years and populations of the same species remains largely unexplained (Petrie and Kempenaers 1998; Griffith et al. 2002), while it strongly suggests a major role for ecological factors in shaping EPP patterns within species (Griffith et al. 2002). For instance, environmental conditions could clump breeding dates and increase the temporal overlap of the reproductive activities of different individuals, thus potentially affecting the occurrence of EPP (Westneat and Steward 2003; Westneat and Mays 2005). Here we highlight the importance of addressing individual behavior in trying to explain variation in population patterns in EPP under specific ecological and social settings. Understanding how individuals make their choices in different conditions (e.g. to prioritize mate guarding vs. extra-pair copulations; EPC) may help to explain why the same population displays contrasting patterns in EPP under contrasting scenarios.

Ecological factors such as breeding density or synchrony have been traditionally proposed as main determinants of EPP rates at the population level (e.g. Birkhead and Biggins 1987; Stutchbury and Morton 1995; Weatherhead 1997; Westneat and Sherman 1997; Stutchbury 1998; Richardson and Burke 2001; Johnsen and Lifjeld 2003; Lindstedt et al. 2007). However, the influence of these factors on EPP rates is not well supported in comparative studies (e.g. Birkhead and Biggins

1987; Stutchbury and Morton 1995; Westneat et al. 1990; Westneat and Sherman 1997; Stutchbury 1998). Likewise, contradictory results also abound in intraspecific studies (Dunn et al. 1994; Weatherhead 1997; Richardson and Burke 2001; Lindstedt et al.2007). A number of studies have found a positive influence of breeding density on the frequency of EPP by enhancing the encounter rate between potential mates (Richardson and Burke 2001; Stewart et al. 2006), while others have not found such an effect (Dunn et al 1994; Tarof et al. 1998). Predictions on the effects of breeding synchrony upon EPP rates may vary depending on the behavioral strategies followed by each sex (Stutchbury and Morton 1995; Westneat and Sherman 1997; Stutchbury 1998). When females pursue EPCs, a high number of fertile females (and hence of displaying males) would tend to increase EPP rates by enhancing the ability of females to simultaneously assess the quality of several potential males (e.g. Stutchbury and Morton 1995; Hoi and Hoi-Leitner 1997; van Dongen and Mulder 2009). Likewise, when EPCs are initiated by males, a high synchrony should raise the probability of encountering fertile females (Stutchbury and Morton 1995). However, the effect of breeding synchrony on EPP rates would depend on whether males prioritize assuring paternity in their social nests over searching for EPP (Birkhead and Biggins 1987; Westneat et al. 1990; Stutchbury and Morton 1995), as they usually are unable to simultaneously maximize paternity outside of and within the pair bond (Kokko and Morrell 2005). Moreover, strategies may differ among males as, for instance, a high concentration of breeding individuals may incite both high quality males to invest more in seeking EPP and low quality males to guard their mates more intensively. Both circumstances would tend to obscure the association between breeding synchrony and population density with EPP (Stewart et al. 2006). Thus, besides differences due to phylogenetic history (Griffith et al. 2002), the understanding of individual behavior (or rather, its lack thereof) may play a major role in the current discrepancies between studies in EPP rates and their interpretation.

Given that EPP emerges from the interaction among at least three individuals − a female, its social pair and the extra pair male − only males and females co-occurring in space and time can eventually engage in EPCs (Westneat and Steward 2003). Thus, focusing on individual behavior demands approaches at the spatiotemporal scales at which individuals make their choices (i.e. select among available possibilities; Chuang et al. 1999; Webster et al. 2001). Importantly, this detailed individual-level information can easily translate to the understanding of population patterns. For instance, if EPP only involves individuals from neighboring territories, the overall breeding synchrony of the population may not shape EPP opportunities of individuals if it does not lead to breeding synchrony between neighbors (Chuang et al. 1999). Spatial and temporal factors could further interact, e.g. a negative relationship between EPP rates and synchrony may only occur under certain breeding densities (Thusius et al. 2001). Moreover, is important to identify the extra pair male(s) in order to consider the breeding status of all individuals involved in EPP. This is because the likelihood of an extra pair fertilization (EPF) will be likely influenced not only by factors affecting the behavior of an individual alone (its costs and benefits), but also by those affecting the behavior of all individuals involved in an EPP event (Westneat 1993; Westneat and Stewart 2003).

Here we studied the distribution of EPP events (EPPs) in two years with contrasting breeding synchrony in a Spanish population of pied flycatchers (Ficedula hypoleuca), a long-distance migrant passerine that establishes a territory around the nest site hole. Occurrence of EPP in this species is relatively common, with rates of extra pair young (EPY) varying across populations by between 4-24 % (Canal et al. 2011 and references therein). Thus, pied flycatchers exemplify the variation of EPP rates among populations and the discrepancies in determining the causes of that variation (Lifjeld et al. 1991; Gelter and Tegelström 1992; Rätti et al. 2001).

We hypothesize a trade-off between mate guarding and seeking for EPCs in males (we assume EPCs are mainly male-initiated, as suggested by previous work in

pied flycatchers, Björklund and Westman 1983; Alatalo et al. 1987). Under this hypothesis, we predict that males will seek for EPCs preferably among neighboring females due to costs (loss of within-pair paternity) derived from searching for EPCs. Temporally, we predict more EPP events after the laying date of the extra pair males' social females as males would invest more in mate guarding during the fertile period of their social female. To test this hypothesis we identified extra pair sires and analyzed the phenology of EPP events relative to the breeding stage of their social female, taking into account the spatio-temporal accessibility to fertile females for each male. Under this hypothesis, we also predict that breeding synchrony would negatively affect EPP rates at the population level. This is because if males invest in mate guarding during the fertile period of their social female a high synchrony of females' fertile period would reduce EPP rates in the population by decreasing the time available for males to engage in EPCs.

MATERIAL AND METHODS

Field work

The study was carried out during two consecutive breeding seasons (2005-2006) as part of a long-term study of pied flycatchers in central Spain (e.g. Potti and Montalvo 1991, Potti and Canal 2011). The study area consists of two plots 1.3 Km apart, including 236 nestboxes (Fig. 1). The plots are located in an old oak (Quecus pyrenaica) deciduous forest and a coniferous stand (mainly constituted by Pinus sylvestris and P. pinaster) with sparse old oaks. UTM coordinates of all nests were GPS-referenced and distances among them calculated with Arcview (ESRITM 2000). Average (SD) distance among occupied nestboxes was 30 (14.1) m.

Field protocols have been described in detail elsewhere (Potti and Montalvo 1991a,b; Potti and Canal 2011). Briefly, all nests were regularly checked every three days before the onset of egg laying and on a daily basis around hatching to ascertain

laying date, clutch size, hatching date and number of fledglings. Parent birds were captured with a nestbox trap while they were feeding eight day-old nestlings. Fledglings were banded at 13 days of age. Blood samples were taken from all individuals by puncturing the brachial vein and stored in ethanol.

Molecular methods

A total of 1,567 individuals were used in parentage analyses: 531 chicks and 212 adults (113 females and 99 males) from 113 nests in 2005, and 595 chicks and 229 adults (120 females and 109 males) from 120 nests in 2006. Within-year discrepancies in male and female numbers are due to bigamous pairings.

Paternity assignments were performed in CERVUS 3.0 (Marshall et al. 1998) using a maximum likelihood method. Individuals from 2005 were genotyped at seven polymorphic microsatellite loci (fhu1 and fhu2 (Ellegren 1992), fhu3 and fhu4 (Primmer et al. 1996) and Fhy6-126, Fhy1-25, Fhy3-60 (Chapter I)). In addition, to increase reliability in the assignment of genetic fathers we genotyped all individuals from nests containing young that showed mismatches with their putative father with three additional primers (fhy444, fhy466 and fhy310; Leder et al. 2008). Individuals from 2006 were genotyped at fifteen microsatellite isolated (f3-60, f1-25 (Canal et al. 2009) and fhy 216, fhy 237, fhy 301, fhy 304, fhy 310, fhy 329, fhy 339, fhy 356, fhy 361, fhy 401, fhy 444, fhy 466 and fhy 236 (Leder et al. 2008); see Chapter V). The combined probability of exclusion for all loci was >99.9%. A nestling was considered as an EPY when the social father was not among the most likely sires given by CERVUS, or when another male showed a significant match with him (see below). A given male was identified as extra pair sire when he had a LOD (natural logarithm of the likelihood ratio) score with an EPY higher than the critical value (which is computed by CERVUS through parentage analyses simulations) requested for assignments at 95 % confidence level. Some nestlings were considered as EPY with

unknown fathers since no male showed a good match with them. Paternity assignments performed at 80 % confidence level did not show any discrepancy with those at 95%, thus confirming both that unknown fathers were not sampled and that our assignments were reliable. See Chapter II for further details on paternity analyses.

Patterns of extra pair paternity

Laying dates were scored as days after the 1st of May. Laying date differences between the social and the extra pair mate of a given male (hereafter ΔLD) were calculated by subtracting the social female's laying date from that of the extra pair female. For instance, ΔLD was + 5 days for a male whose social and extra pair females laid their first egg (day 0) on 15th and 20th of May, respectively. Likewise, we calculated the difference in laying dates and the linear distances between all breeding pairs in the population. An index of synchrony (SI; Kempeaners 1993), indicating the average proportion of fertile females per day in the population, was also calculated for each year.

Female birds can store sperm up to several weeks, although early EPCs have a reduced chance of success due to last-male sperm precedence (Birkhead and Møller 1992; Birkhead 1998; Michl et al. 2002). In pied flycatchers, however, females seem to store sperm only from day -2 onwards (Birkhead et al. 1997). Thus, we define the fertile period as starting at day -2 until the day the penultimate egg was laid (Birkhead and Møller 1992; Lifjeld et al. 1997; Birkhead 1998). Moreover, the highest insemination rate in pied flycatchers and its sister species, the collared flycatcher (*Ficedula albicollis*), occurs between days -2 and +1 (Lifjeld et al. 1997; Michl et al. 2002). In fact, Lifjeld et al. (1997) showed that male pied flycatchers removed from their territories before day -3 did not sire any young in the clutch whereas those removed on day +1 fertilized the entire clutch. Therefore, we assumed that most

inseminations should have occurred during days -2 to +1 and thereby that an EPP event is an accurate proxy of the moment wherein an EPC occurred.

The secondary status of a brood may affect paternity of the offspring if males spend less time potentially guarding females during their fertile period (Lundberg and Alatalo 1992). To confirm that data from secondary females engaging in EPP (2005: 4 out of 14 cases; 2006: 1 out of 11) did not bias our conclusions we repeated all analyses excluding the cases of secondary females engaging in EPP but results remained unchanged (data not shown).

Spatiotemporal patterns and EPP opportunities

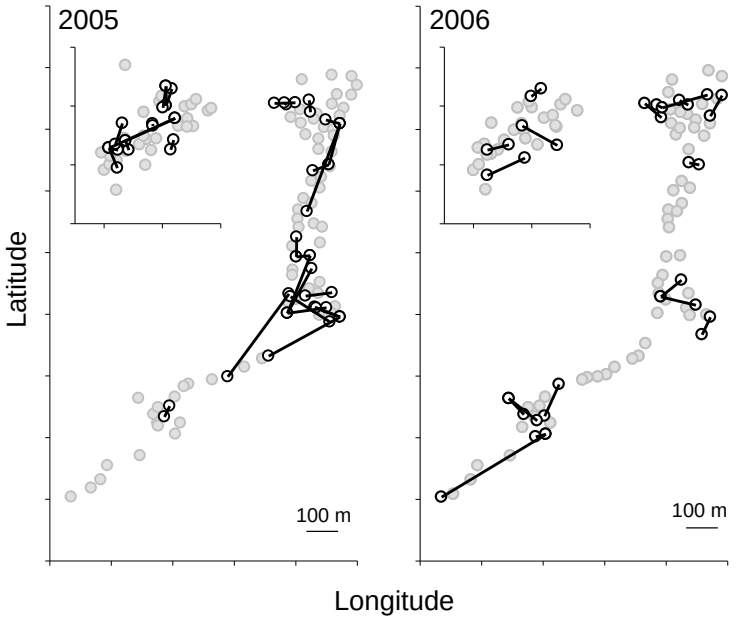
To analyze whether the probability of EPP (presence vs absence of EPY in a nest) was spatially influenced, we coded the distance between nests (obtained from pairwise comparisons; see above) by stretches of 30 m (i.e. the average distance between nests in the population). Thus, for a given focal breeding pair, all pairs breeding at distances lower than 30 m were included in group 1, those from 31 to 60 m in group 2, etc. Then, the probability of EPP between two nests of a given stretch (number of EPPs / number of breeding pairs) was modeled with a generalized linear model (GLM) with binomial distribution and the midpoint distance of the section as an explanatory variable.

The probability of EPP (presence vs absence of EPY in a nest) in relation to the number of accessible females was modeled with a generalized linear model with binomial distribution. We only considered as accessible females for a given male those fertile females (see above) breeding within the spatiotemporal scale (i.e. distance between nests and ΔLD) at which EPPs occurred in each year (see Results).

We also tested whether the distribution of EPPs relative to the breeding status of each extra pair male's social female was the consequence of a male's strategy to maximize paternity (by mate guarding before their social female's egg laying and

engaging in EPP afterwards) or if, in contrast, EPPs were a mere outcome of female accessibility. For each male, the ΔLD and distance in relation to each female in the population were computed and only accessible females for each male were considered. Finally, we tested with Fisher's exact tests whether observed and expected frequencies of realized EPP differed from those of accessible females before and after the social female's egg laying onset.

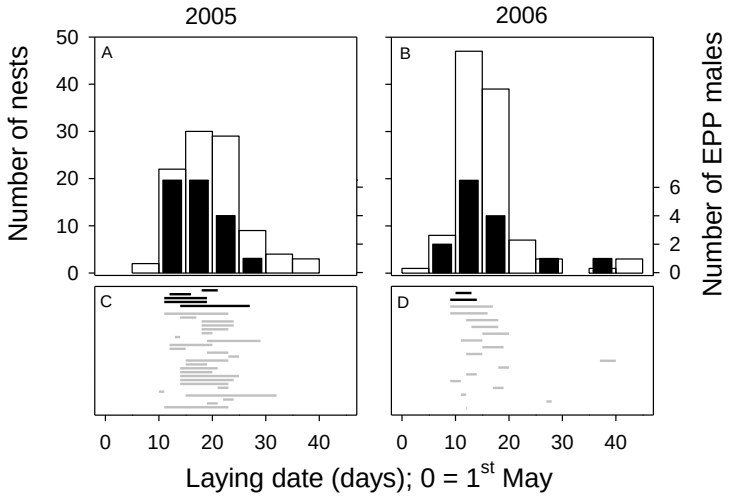
Figure 1. Map of the study area (inset small panels correspond to an area 1.3 Km north-east to the main panels). Black circles (linked by lines) indicate territories involved in EPP, whereas gray circles represent breeding pairs without EPP. Empty territories in each year are not represented.



Data from both study plots were grouped for analyses. This is justified because breeding synchrony was consistently higher in 2006 than in 2005 in both areas (2005: 29.8 % and 28.6 % in the oak and pine plots, respectively; 2006: 40.5 % and 52.9 %). Also, neither breeding density (2005: 8.5 and 8.1 pairs/ha, p= 0.81; 2006:

8.5 and 7.5 pairs/ha, p= 0.52), distance between EPP mates (GLM: $\chi^2_1 = 1.74$, p = 0.18 and $\chi^2_1 = 0.23$, p = 0.63 in 2005 and 2006, respectively) or frequency of EPP events (Fisher's exact tests: p = 0.36 and p = 0.22) differed between plots. Statistical analyses were done in SAS 9.1 (SAS Institute 2004) and Statistica 7.

Figure 2. (A-B) Frequency distribution of all laying dates in the population (white bars, left y-axis) and laying dates of the social female of those males involved in EPP (black bars, right y-axis). (C-D) Lines link the laying dates of the social female of males engaging in EPP to those of their extra pair female(s); black lines stand for males that engaged in EPP before their social females started to lay whereas gray lines indicate males engaging in EPP after their social females laying date.

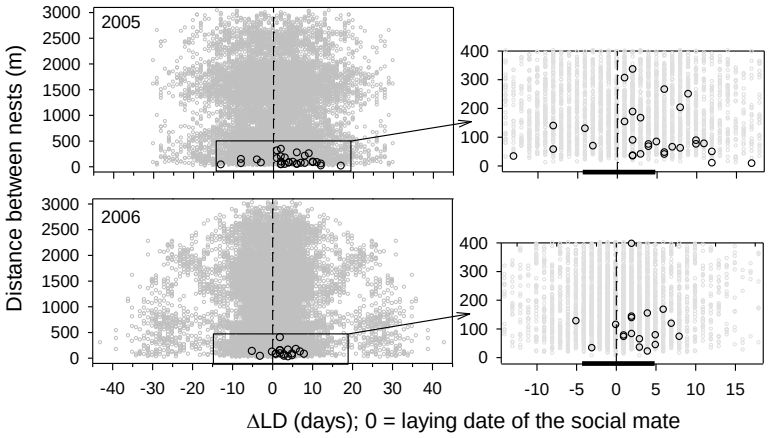


RESULTS

In 2005, 40 % (N = 113) of the nests and 33 % (N = 212) of the adults were involved in EPP, with 20 % (N = 531) of the offspring being EPY. Respective figures in 2006 were lower than in 2005: 27 % (N = 120; $\chi^2_1 = 4.55$, p = 0.03), 21 % (N = 229; $\chi^2_1 = 8.17$, p = 0.04) and 11 % (N = 595; $\chi^2_1 = 16.64$, p = 0.001). The genetic father was

identified for 67 % (N = 106) and 66 % (N = 68) of the EPY in 2005 and 2006, respectively. Breeding synchrony was higher in 2006 (SI = 39.7 %) than in 2005 (SI = 27.7 %, Fig. 2A, B).

Figure 3. Spatial (distance) and temporal (difference in laying dates, ΔLD) relations between pairs of individuals involved (black circles) or not (gray circles) in EPP at the population level (left panels) and at the spatiotemporal scale at which interactions occurred (right panels). Bold lines on the x-axis of the right panels indicate the fertile period of the extra pair's social females.



Spatiotemporal patterns in EPP at the population level

Females engaging in EPP laid consistently later than the females of their extra pair males: an average (range) of 3.9 (-13, +17) days later in 2005 (Paired t-test: t = 3.06, df = 29, p = 0.004) and 2.7 (-5, +8) days in 2006 (t = 2.8, df = 17, p = 0.012). In other words, males usually attained EPP after their social female had started to lay (Fig. 2C, D and Fig. 3). This occurred in 83 % (N = 30) and 88 % (N = 18) of the EPPs in 2005 and 2006, respectively. Spatially, EPPs occurred on average (range) at 107 (17-334) m and 99 (18-395) m from the social nest, in 2005 and 2006, respectively

(Fig. 1 and 3). Thus, overall, EPPs occurred at a shorter spatiotemporal scale than that imposed by breeding phenology (Fig. 2) or the extension of the study area (Fig. 1 and 3).

EPP patterns within realized spatiotemporal scales

Within the spatiotemporal scale (see above) at which EPP interactions occurred, the probability of EPP decreased with the distance between nests (GLM: $\chi^2_1 = 38.01$, $p < 0.001$; Fig 4a). However, at the individual level, 75 % of the males did not engage in EPP with the closest accessible fertile female, but did so with females breeding at more distant territories in both years (median (range) = 5th (1-29) and 3rd (1-23) territory in 2005 and 2006, respectively; Fig. 5).

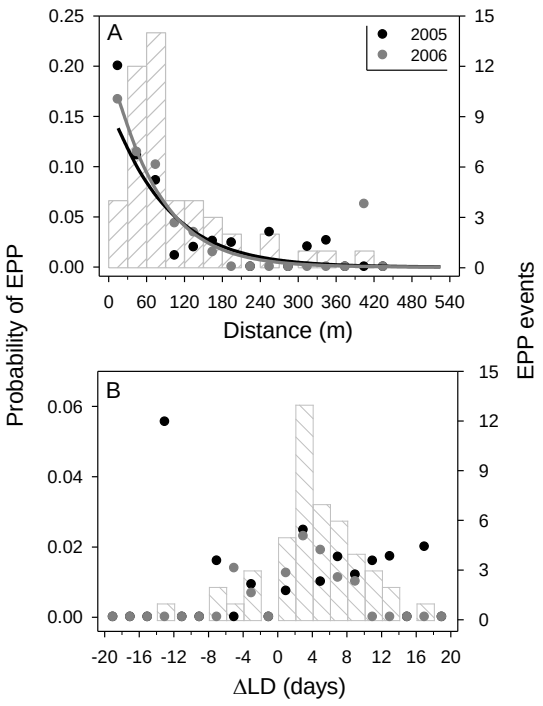
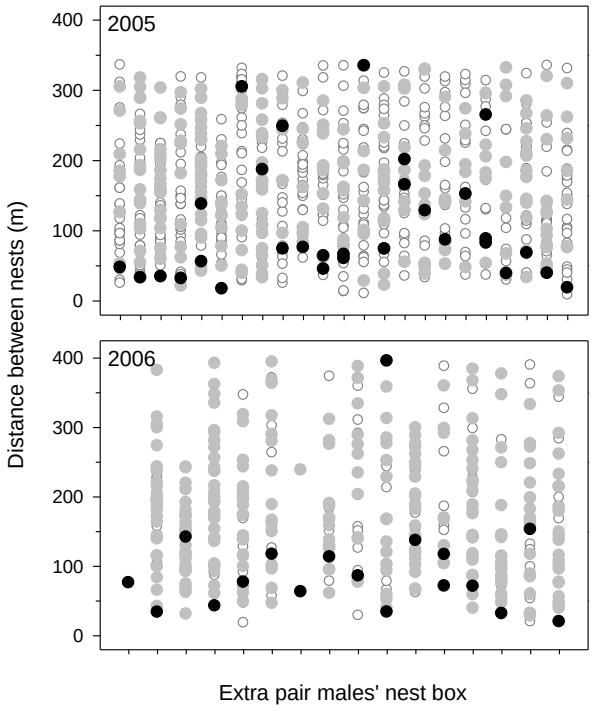


Figure 4. Probability (dots) and number of EPP (bars) events in relation to (A) the distance between nests, and (B) the difference in laying dates (ΔLD) between the nest of the male and the female engaging in an EPP. EPP probability = number of EPPs / number of breeding pairs in each stretch of 30 m or period of two days.

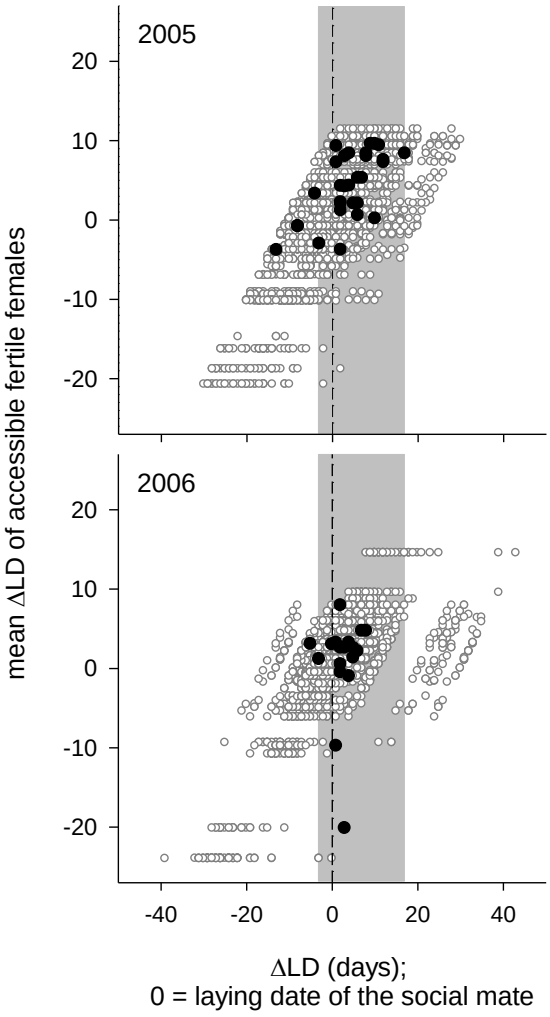
Figure 5. Spatial distribution of the EPP events (black circles) in relation to the accessible mates for extra pair males. White circles indicate accessible females whereas gray circles indicate highly synchronous females with respect to the extra pair male's social female (i.e. -3 to + 4 days).



Temporally, the probability of EPP was strongly dependent on the number of accessible fertile females in both years (GLM: $\chi^2_1 = 71.32$, $p < 0.001$; year*number of accessible females: $\chi^2_1 = 0.58$, $p = 0.44$). When both the number of accessible females and the breeding status of the extra pair male's social female were considered, most of males that attained EPP did so once their social females had already laid the first egg (Fisher's exact tests: $p = 0.004$ and $p = 0.044$ in 2005 and 2006, respectively) despite there were accessible females before those dates (Fig. 6). Moreover, the frequency of EPP decreased during the days prior to the social female's laying date (none of 48

EPPs occurred in days -2 and -1) while 85% of them occurred during the egg laying and incubation periods (Fig. 4b and 6) and none afterwards, suggesting that males were engaged in chick rearing afterwards.

Figure 6. Temporal distribution of EPP (black dots) and accessible females for males (gray dots) in relation to their social female's laying date. The gray area indicates the fertile and incubation period of a male's social female (i.e. -3 to +17 days).



DISCUSSION

The probability of EPP at the population level was temporally tied to the number of accessible fertile females. Spatially, the occurrence of EPP decreased with distance between nests. As a consequence, EPPs occurred at a shorter scale than that possible according to the population breeding phenology and spatial extent. Most EPPs occurred after the social females of extra pair males had started laying (i.e. after their peak of fertility) and before the eggs hatched. These patterns suggest a male's strategy to maximize paternity by guarding their females during the critical period of inseminations, searching for EPC during egg laying and incubation and focusing on rearing their chicks upon hatching. At the population scale, therefore, our results suggest that, spatially, individuals tried to remain as close as possible to their nests when engaging in EPP and, temporally, that EPP was (obviously) restricted by breeding phenology. However, when simultaneously taking into account the individual accessibility to fertile females and the scale at which EPPs occurred (i.e. at the local scale) the spatial picture changed as males usually did not engage in EPP with the closest accessible females.

The accessibility to fertile females shaped the distribution of EPPs along the breeding season. The between-year differences in EPP rates could thus be explained by differences in synchrony since a more synchronous breeding season (i.e. 2006 as compared with 2005; Fig. 2A, B) should impose additional time constraints on EPP. This is because males face a conflict over paternity, as the chances of gaining it (e.g. through exploratory behaviors for EPCs) reduce those of lowering cuckoldry in their own nests (e.g. through mate guarding) and both activities can hardly be simultaneously maximized (Hasselquist and Bensch 1991; Kokko and Morrell 2005). Thus, when EPCs are mainly male-initiated, as suggested by previous work in pied flycatchers (Björklund and Westman 1983; Alatalo et al. 1987), a negative relationship between synchrony and EPP rates would occur when males prioritize avoiding

cuckoldry over seeking paternity outside the pair bond (Birkhead and Biggins 1987; Westneat et al. 1990). Accordingly, when the risk of cuckoldry is high, fairy martin males (*Petrochelidon ariel*) guard their mates more intensively (Hammers et al. 2009) whereas golden whistler males (*Pachycephala pectoralis*) are more aggressive towards intruders and remain closer to their mates (van Dongen 2008).

Most EPPs occurred while the extra pair male's social females were laying or incubating, despite the presence of a great number of fertile females before and after this period (Fig. 6). This strongly suggests that males favored both securing their paternity and engaging in parental duties in their social nests over gaining paternity in other nests. However, the key factor here is that successful males in EPP usually bred early in the season (Chapter III; Fig. 2C, D) and thereby many females were still fertile after their social females' laying onset. Successful males in EPP could thus have solved the conflict over paternity since when female's fertility is asynchronous within the population, guarding the social female during her fertile period and searching afterwards for additional paternity seems to be an evolutionarily stable strategy (Birkhead and Biggins 1987; Kokko and Morrell 2005). For instance, wood thrush males (*Hylocichla mustelina*) search for EPC after the fertile period of their social females (Evans et al 2008) and experimentally induced late broods of house sparrows (*Passer domesticus*) contained more EPY sired by early males whose females were already incubating (Václav and Hoi 2007). Therefore, a high synchrony may lessen the population rate of EPP by decreasing the effective time to attain it but, at the same time, may increase the variance in EPP opportunities between males, since those breeding early relative to their neighbors should enjoy more EPP opportunities (and lower costs of cuckoldry) than the other males (Birkhead and Biggins 1987; Václav and Hoi 2007). However, it could also be argued that protandry (often associated with earlier breeding) may have evolved because of the benefit males gain by increasing female accessibility after their social females lay, thus boosting their chances of EPCs (Coppack et al. 2006; Chapter III). From a female's point of view, engaging in EPP

with early males could report some type of either direct (Lozano et al. 1996) or genetic (Akçay and Roughgarden 2007) benefit, as arrival date is often reported as being a reliable signal of male quality in migrant birds of temperate regions (e.g. Lozano et al. 1996; Smith and Moore 2005). We emphasize that accounting for the timing of breeding relative to others may increase our ability to comprehend individual decisions related to EPP and thereby the effects of breeding synchrony upon EPP rates at the population level.

Remarkably, the frequency of EPP dropped in the days previous to the extra pair males' social female laying onset (days -2, -1). Despite this could be taken as anecdotal evidence, it is also in agreement with a males' decision about when it would pay to pursue EPCs (Birkhead and Biggins 1987; Birkhead 1998). This is because those are the days when most fertilizations occur in birds (see Birkhead and Møller1992 for a review) and thereby when males should increase their efforts to avoid loss of paternity. Thus, for instance, red-winged blackbird (*Agelaius phoeniceus*) males copulate more often during the days prior to the onset of egg laying (Westneat 1993) and in superb fairy wrens (*Malurus cyaneus*), a species where females foray outside the territory more commonly during their peak of fertility, males more intensively pursue their mates (Double and Cockburn 2000). Accordingly, previous studies in pied flycatchers have shown that males seem to prioritize mate guarding before egg laying (Björklund and Westman 1983) since most fertilizations occur in days -2 and -1 (von Haartman 1956; Alatalo et al. 1987; Lifjeld et al. 1997b) and males experimentally switched in day +1 fertilized the whole clutch (suggesting that inseminations fertilizing last eggs occur several days before; Lifjeld et al. 1997b). However, the effectiveness of mate guarding is uncertain (Birkhead 1998; Stutchbury and Neudorf, 1998) since there is evidence showing that females may sometimes circumvent the constraints imposed by male behavioral strategies (e.g. Kempenaers et al. 1995; Johnsen et al.1998). We cannot discard the possibility that the low

probability of EPP observed before egg laying could also be due to other factors such as aggressiveness among females (Slagsvold et al. 1999).

Further evidence for EPP being male-initiated comes from the absence of EPPs after nestlings hatch in social nests, despite sustained opportunities for additional matings. At the same time, this also supports the idea that engaging in EPP is a costly behavior. In fact, investment in EPP is expected to trade-off against parental care duties (Magrath and Komdeur 2003). For instance, pursuing EPCs could have a large negative impact on nestling fitness (and hence, on male reproductive success), especially in the early stages of nestling development, if such behavior implies a reduction in males' chick-feeding rates due to the time spent away from the territory (Magrath and Komdeur 2003).

Spatially, the pattern of EPP found here contrasts with that most common in passerines, wherein extra pair males usually are the nearest or the next-to-nearest neighbors (e.g., Gibbs et al. 1990; Kempenaers et al. 1992; Stutchbury et al. 1997; Yezerinac et al. 1995; Freeman-Gallant et al 2005; Pedersen et al. 2006; van Dongen and Mulder 2009). However, it is similar to that found in scarlet rosefinches (*Carpodacus erythrinus*; Albrecht et al. 2007), red-winged blackbirds (Westneat and Mays 2005) or in a northern population of pied flycatchers (Rätti et al. 1995; but see Björklund and Westman 1983). Several non-mutually exclusive circumstances could explain why males often did not attain EPPs with the closest accessible females. For instance, a low willingness of the neighboring female to accept EPCs (e.g. if they are mated with a higher-quality male) or an effective behavior (e.g. mate guarding) of the social mate to prevent loss of paternity could explain this pattern. Experimental approaches addressing male/female readiness to engage in EPC (e.g. in relation to social mate presence; Lindstedt et al. 2007) and radio-tracking studies (Kilpimaa et al. 1995; Pedersen et al. 2006) are needed to improve our understanding of the population consequences of variation in individual behavior upon EPP spatio-temporal patterns.

In sum, EPP was not constrained to closest neighbors. Despite often having females closer to them, males sired young up to 390 m away from their nests, implying a high mobility of individuals. Variation in the time window of accessibility to fertile females was a major factor underlying patterns in EPP. A high proportion of extra pair males gained paternity during the egg laying and incubation periods of their social females, despite the fact that there were fertile females accessible before and afterwards. These patterns suggest a male's strategy to optimize paternity through EPP and provide an explanation for why the year with higher breeding synchrony was the year with lower EPP occurrence. Therefore, our work encourages studies on EPP to be carried out at the spatiotemporal scale at which the individual behavior takes place while simultaneously considering the social contexts of all players involved in an EPP event.

CAPITULO V

Exploring heterozygosity survival correlations across different life stages and contexts in pied flycatchers

Pollos de papamoscas al eclosionar y al abandonar el nido, aproximadamente a los 15 dias

Fotos: Carlos Camacho y David Canal

Canal D, Vögueli M, Potti J. Survival is not related to heterozygosity in pied flycatchers. Submitted.

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ABSTRACT

Understanding the relationship between genetic diversity and fitness is a major concern in evolutionary and conservation biology. This relationship is expected to be stronger in traits affected by many loci and those that directly influence fitness. Here we explore the influence of heterozygosity measured at 15 neutral markers on individual survival, likely the most important parameter determining individual fitness. Taking differences in recapture probability into account, we followed individual survival up to recruitment and during subsequent adult life of 863 fledgling pied flycatchers born in two consecutive breeding seasons. Mark-recapture analyses showed that heterozygosity was not associated with both juvenile and adult survival. The lack of relationship between heterozygosity and survival was not context dependent because it was unaffected by hatching date, ectoparasitic (mites and blowflies) load in natal nests or social status of the brood (according to the mating status of the female), all of them factors potentially affecting survival. Furthermore, neither maternal and paternal heterozygosity nor their interaction with other variables influenced fledgling survival. The absence of heterozygosity fitness correlations found here could be due to strong selection occurring at earlier stages than those measured here, to stochastic factors affecting fledgling survival, or because the measured loci were unrelated to loci associated with survival.

Keywords: Heterozygosity, juvenile survival, lifespan, context-dependence, capture-recapture models, Ficedula hypoleuca.

INTRODUCTION

Mating between related individuals usually causes costs in fitness to their descendents (e.g. Charlesworth and Charlesworth 1987, 1999; Lynch and Walsh 1998). The relationship between genetic diversity and fitness has therefore received much attention due to its potential importance in animal production, conservation or evolutionary biology (review in Hedrick and Kalinowski 2000; Hansson and Westerberg 2002; Keller and Waller 2002; Coltman and Slate 2003; Kempenaers 2007; Chapman et al. 2009). The study of this relationship in natural populations has traditionally been complex due to the difficulty of generating pedigrees to measure individual coancestry (Keller and Waller 2002). With the expansion of molecular techniques in the last decades, however, an alternative approach based on the relationship between genetic diversity measured at a set of loci and traits related to fitness (heterozygosity-fitness correlation, HFC) has become widespread in literature (review in Coltman and Slate 2003; Chapman et al. 2009; Szulkin et al. 2010).

Positive HFCs, the association most commonly found in the literature (Chapman et al. 2009), may arise mainly through three mechanisms (David 1998; Hansson and Westerberg 2002). Under the "direct hypothesis" heterozygous individuals have higher fitness due to overdominance of the typed loci. This mechanism may be important when heterozygosity is measured with allozymes or major histocompatibility complex (MHC) loci, but does not explain HFC in studies using microsatellites which, aside from exceptions (e.g. Olano-Marin et al. 2011b), are considered neutral loci (Jarne and Lagoda 1996). When employing microsatellites, two alternative hypotheses have been proposed to explain HFCs. First, the "local effect hypothesis" predicts that HFCs arise indirectly because the typed loci are linked to functional loci influencing fitness. Genetic drift, migration and selection generate linkage favoring the detection of local effects (Lynch and Walsh 1998; Szulkin et al. 2010). Although linkage is expected to be low and rapidly eroded by recombination in

natural populations (Lynch and Walsh 1998; Szulkin et al. 2010), previous work has shown that relatively high levels of linkage may exist (Reich et al. 2001; Dawson et al. 2002) and be maintained after more than 800 generations following a bottleneck (Reich et al. 2001) in natural populations. Second, under the "general effect hypothesis", heterozygosity measured at multiple loci (MLH) reflects heterozygosity across the genome. In such cases, homozygous individuals suffer fitness costs due to their higher likelihood of expression of deleterious recessive alleles (inbreeding depression; Charlesworth and Charlesworth 1987, 1999). This mechanism is expected to arise under random mating, in populations with genetic drift, population admixture or suffering from recent bottlenecks, or in large populations where consanguineous matings occur. Populations under these conditions exhibit a large variance in inbreeding values, which determine the strength of the relationship between MLH and fitness (Balloux et al. 2004, Slate et al. 2004).

Evidence of HFCs is widespread but also inconsistent in the literature (e.g. Hansson et al. 2001, 2004; Acevedo-Whitehouse et al. 2006; Rijks et al. 2008; Wetzel et al. 2011; Chapman and Sheldon 2011; Forstmeier et al. 2012). In general, HFCs are weak signals explaining no more than 3.6 % of the variance in fitness (Chapman et al. 2009). Nevertheless, the magnitude of HFC may depend on the characteristics of the population (see above), the traits under scrutiny, and the environmental conditions that individuals experience (Slate et al. 2004; Armbruster and Reed 2005; Szulkin et al. 2010). HFCs have been commonly explored in behavioral (e.g. song complexity: Marshall et al. 2003) or morphological traits (e.g. attractiveness: Foerster et al. 2003; body size: Ryder et al. 2010), which are often under stabilizing selection (Houle et al. 1996). However, evolutionary theory predicts (Houle et al. 1996), and empirical work confirms (Coltman and Slate 2003; but see Chapman et al. 2009), greater HFCs in fitness-related traits under directional selection that are affected by multiple loci susceptible of deleterious recessive mutations. This is the case of life history traits (e.g. fecundity, lifetime reproductive success, survival), although even life history traits may

have a certain degree of plasticity that may obscure HFCs (Szulkin et al. 2010). Survival is likely one of the most important factors determining individual fitness and evidence of heterozygosity-survival correlations (HSC, hereafter) is common (Coulson et al. 1998; Da Silva et al. 2006; Acevedo-Whitehouse et al. 2006). In birds, there is increasing information on HSC during early life (embryonic and nestling life, or up to recruitment: Olano-Marin et al. 2011a, b; Hansson et al. 2001, 2004; Jensen et al. 2007). Nevertheless, HSC has only been explored beyond those stages, to our knowledge, in the Seychelles warbler (Acrocephalus sechellensis; Richardson et al. 2004; Brouwer et al. 2007), most likely due to the difficulties of researchers to assess individual lifespan in free-ranging populations.

HFCs are expected to decrease with age since differences in survival are maximal in early life (David 1998). Studying HFCs across the lifespan, however, is essential because inbreeding effects may be underestimated (Szulkin et al. 2007; Grueber et al. 2010) or even undetectable (von Hardenberg et al. 2007) when analyzed at a single stage. In addition, MLH effects may be negative early in life but positive during adult life (Olano-Marin et al. 2011a). In combination with age, the role of the genetic diversity on fitness may also be sensitive to the environmental conditions that individuals experience (Keller and Waller 2002; Armbruster and Reed 2005). Accordingly, recent studies highlight that, as a consequence of context-dependence, the magnitude of HFC may be inconsistent across years (Harrison et al. 2011) or undetectable under favorable environmental conditions (Lesbarrères et al. 2005, Brouwer et al. 2007), i.e. if variance in the measured trait is affected by the environment, HFCs will be more easily detected in periods when environmental conditions cause high variation in the trait.

Here, we investigated the relationship between heterozygosity and individual survival across different life stages in a population of pied flycatchers (Ficedula hypoleuca), a long-distant passerine migrant. To explore individual survival we used capture-recapture methods. This modeling framework is a more robust approach than

generalized linear models when exploring effects on survival in open populations because it accounts for left truncation and resighting probabilities (Lebreton et al. 1992, Kalbfleisch and Prentice 2002). Specifically, we explored whether: i) variation in survival was influenced by individual heterozygosity (MLH and/or single loci heterozygosity) or by the heterozygosity of an individual's parents, and ii) MLH and/or the magnitude of HFC changed across lifetime (David 1998). Furthermore, we accounted for factors which may potentially affect individual survival of pied flycatchers (see below). Thus, this study provides an excellent opportunity to investigate the influence of individual heterozygosity on survival in a wild population throughout juvenile and adult life stages and under a range of environmental contexts.

MATERIAL AND METHODS

Field work and general procedures

The study was carried out with individuals born in the breeding season of 2005 and 2006 as part of a long-term study of pied flycatchers in central Spain (e.g., Potti and Canal 2011; Canal et al. 2011). The study area consists of two plots separated by 1.3 km, including 236 nest-boxes. Field protocols have been described in detail elsewhere (Canal et al. 2011). Briefly, all nests were regularly checked every three days before the onset of egg laying and on a daily basis around hatching to ascertain laying date, clutch size, hatching date and number of fledglings. Parent birds were captured with a nest-box trap while feeding eight day-old nestlings. They were weighed, measured and individually marked with a numbered metal band and a unique combination of colored bands. Fledglings were banded, measured and weighed at 13 days of age. Blood samples were taken from all fledglings by puncturing the brachial vein and stored in absolute ethanol. Sex determination was carried out by PCR amplification of the CHD gene using the primers 2917 (forward) and 3088 (reverse; Ellegren 1996). Molecular sexing was always fully consistent with the sex of recruited individuals.

Apparent survival of individual offspring was assessed through an extensive effort of marking, recapturing and resighting of color-banded birds in all subsequent breeding seasons until 2011. This population has high natal philopatry with a mean of 13% of recruitment of locally born birds, which is the highest recruitment rate found so far for the species (Potti and Montalvo 1991, Lundberg and Alatalo 1992; J. Potti and D. Canal unpubl. data).

The species has a predominantly monogamous mating system (Lundberg and Alatalo 1992) although a number of males (12% and 9% in 2005 and 2006, respectively; Canal et al. 2012) acquire a second female after mating and become socially polygamous. We defined as secondary females those assisted by males previously captured (or observed) while feeding nestlings in a different nest. The primary brood of a polygamous male was set as that with the earlier laying date. Secondary females commonly receive less male assistance in feeding nestlings, entailing lower fledgling and recruitment success than those from monogamous or primary females (Alatalo and Lundberg 1984, Lundberg and Alatalo 1992, Potti and Montalvo 1993). For this reason, four categories of mating status were assigned to the females (and accordingly to their broods, hereafter "brood status") during the nestling period: 1) Females of monogamous males, 2) primary females of polygamous males, 3) secondary females of polygamous males, and 4) females without any male assistance. These latter birds could have been secondary females, have been deserted by their mates, or became widowed after pairing, and were grouped together.

Previous studies in the population have shown that ectoparasite load in nests affects fledgling growth and/or survival (Merino and Potti 1995, 1998). Thus, the abundance of ectoparasitic nest mites (Dermanyssus spp.), which may range from zero to thousands, was visually estimated the day when fledglings were measured (Merino and Potti 1995). The abundance of blowflies (Protocalliphora azurea) was also recorded by dismantling the nest material shortly after the young fledged and counting the number of fly larvae and/or pupae (Potti 2008).

Molecular methods

Our data set comprised 868 individuals born in 2005 and 2006. Fledglings were genotyped at 15 polymorphic microsatellite loci: f3-60, f1-25 (Canal et al. 2009), fhy 216, fhy 237, fhy 301, fhy 304, fhy 310, fhy 329, fhy 339, fhy 356, fhy 361, fhy 401, fhy 444, fhy 466 and fhy 236 (Leder et al. 2008). Most individuals (n = 835, 96 %) were genotyped at all loci, whereas genotyping procedure failed for 23 individuals (2.6%) at one locus and for five individuals (0.6%) at two loci, respectively. Five individuals (0.6 %) were discarded for further analyses since they were not genotyped for 4 or more loci.

Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were done using the genotypes from the adult population of each study year in the program Genepop 4.0 (Raymond and Rousset 1995). We performed a search in the zebra finch (Taeniopygia guttata) genome (Warren et al. 2010) to find the chromosome location of the used loci. A BLAT and BLAST search were run in UCSC (http://genome.ucsc.edu/) and ENSEMBL (http://www.ensembl.org/Taeniopygia_guttata/blastview) browsers, respectively, to confirm the locations of the sequences. The best matched sequence was selected on based to both the lowest E-value and highest score. The "contig view" option in ENSEMBL was used to locate the nearest gene to the best matching sequence.

Parentage analyses are detailed in Canal et al. (2011, 2012). They were carried out on CERVUS 3.0 (Marshall et al. 1998) using a maximum likelihood method. We considered a given male as the sire when he had a LOD (natural logarithm of the likelihood) score with a fledgling higher than the critical value requested for assignments at 95 % confidence level (critical value is computed by CERVUS through parentage analyses simulations). Analyses repeated at 80% confidence did not show discrepancies with those at 95%.

Estimation of heterozygosity and identity disequilibrium

Mutilocus individual heterozygosity and allele frequencies were calculated with the Excel macro Cernicalin (Aparicio et al. 2006). Cernicalin calculates three metrics: observed homozygosity per individual (HO), Internal relatedness (IR) and homozygosity by loci (HL). The three metrics were highly correlated ($n = 863$, all $r > 0.97$, $p < 0.001$) and results did not vary among metrics (not shown). Analyses with HL are reported here because HL correlates better with genome-wide homozygosity and inbreeding in open populations than do other metrics (Aparicio et al. 2006). Homozygosity at a single locus (SHL) was coded as "0" for heterozygous status and "1" for homozygous. Previous to analyses, we tested for differences in individual MLH between years and sexes, and their interaction. GLMMs with a normal distribution of error and an identity link function were used, controlling for the nest-box identity due to the non-independence of siblings reared in the same nest-box.

Identity disequilibrium, the positive correlation between heterozygosity across loci, which is expected when MLH is related to wide-genome heterozygosity, was calculated as g_2 in the program RMES (David et al. 2007). Genotypes were resampled 1000 times to test if g_2 differed significantly from zero.

Pedigree information

We reconstructed the pedigree of the whole population from field data obtained since 1987. We visualized the full pedigree and calculated inbreeding coefficients with the program Pedigree viewer (Kinghorn and Kinghorn 2006). Inbreeding values based on grandparents-grandsons are highly correlated with pedigree relationships based on 50 generations because recent inbreeding events have greater impact on individual inbreeding coefficients than events deeper in the pedigree (Balloux et al. 2004). The rate of immigration of the population is high because 53% of individuals breeding

each year were born outside the study area (J. Potti and D. Canal, unpublished data). The ancestral information for these individuals is therefore unknown. As a consequence, the grandparents of nestlings from only 15 broods were identified in the two study years, and the inbreeding value was zero in all cases.

Capture-Recapture models

To analyze the apparent survival we used mark-recapture models in MARK 6.0 (White and Burnham 1999). Survival probability was denoted φ, and reencounter probability was p. Interaction terms were denoted by asterisks when more than one variable was included. We started the analysis with a fully time-dependent Cormack-Jolly-Seber (CJS) model and tested the goodness of fit (GOF) of our data using U-CARE 2.3.2 (Choquet et al. 2009) since CJS models make some fundamental assumptions (Lebreton et al. 1992). The general model had sex- and year-specific probabilities of survival and reencounter (φ(sex*t) p(sex*t)). Then, we also assessed the adjustment of the CJS model to the data with a parametric bootstrap approach in MARK. Parametric estimates (1000 repetitions) from the model were used to simulate data according to the assumptions (independence of individuals and no occurrence of overdispersion of data) contained in the CJS models. Subsequently, we calculated the overdispersion parameter ĉ as the ratio between the deviance of the observed model and the mean deviance of the simulated models (Cooch and White 2004). The fates of individual birds were considered as independent of each other although some overdispersion (ĉ = 1.725) occurred (Anderson et al. 1994). We used a quasi-likelihood corrected model selection criteria for small sample size (QAICc, Burnham and Anderson 2002) for model selection, taking into account overdispersion by adjusting the results to the ideal ĉ = 1.000. However, this adjustment had no qualitative effects on the results. The model with the smallest QAICc was chosen as the most parsimonious model to make inferences about the correspondent

hypotheses. The CJS model did not adequately fit our data because the presence of transient or emigrant individuals was detected (see Results). Transients have zero probability of recapture in subsequent occasions and can negatively bias survival estimates when mixed with residents in the data set (Pradel et al. 1997). Hence, we separated the first and subsequent encounters in the model structure (Pradel et al.1997; Choquet et al. 2005), and continued with a "transient" version of the CJS model.

After establishing our basic general model (based on variation in time and between sexes), we created a set of models with additional variables potentially affecting survival based on previous biological knowledge of the study species (Potti and Montalvo 1991a; Merino and Potti 1995; Potti et al. 2002), and compared them to a basic general model. These models contained brood status introduced as a categorical variable and brood size, hatching date (standardized by year), fledgling body mass, tarsus length and abundances of nest mites and blowflies introduced as continuous variables. In addition, we assessed the influence of genetic effects (SHL and parental MLH) on individual survival. Because our data set contained fledglings resulting from extra pair copulations (20% and 11% of extra pair young in 2005 and 2006, respectively; Canal et al. 2012) we could analyze the influence of genetic effects on both groups of fledglings. The quadratic effects of body mass, tarsus length and MLH were also tested.

For confirmation of single locus effects on survival, we additionally built i) a model including all single locus heterozygosities (SHL), and ii) a model including MLH in a generalized linear modeling framework using a GLMM with a binomial error distribution, and compared them with a F-ratio test as suggested by Szulkin et al. (2010). In the model containing heterozygosities at all single loci, missing data at one locus were replaced by the sample average at that locus (Szulkin et al. 2010). The procedure was performed for both juvenile and adult survival regardless of whether or not general effects were found. In fact, significant SLH effects may exist even in

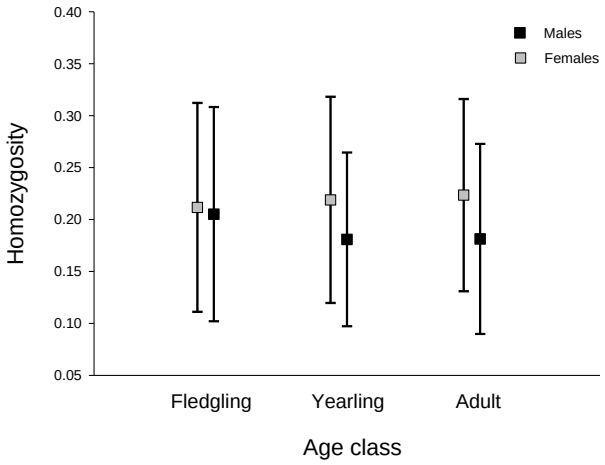
absence of MLH correlation as loci may have similar effects in opposite directions (Chapman et al. 2011). Sample sizes varied slightly across statistical analyses because all information was not always available for all individuals.

RESULTS

General genetic parameters

Individual MLH (n = 863) ranged from 0 to 0.56, with a mean (± standard deviation) of 0.208 (± 0.102). MLH did not differ between breeding seasons (2005, n = 234: 0.198 ± 0.102, 2006, n = 620: 0.212 ± 0.102, GLMM: F1, 666 = 1.10, p = 0.29), individual sex (males, n = 477: 0.205 ± 0.103, females, n = 377: 0.212 ± 0.100, GLMM: F1,665 = 0.70, p = 0.40) or groups of ages (fledglings: 0.21 ± 0.1; yearlings: 0.20 ± 0.09; older individuals: 0.20 ± 0.09; F1, 155 = 0.11, p = 0.89; interaction age*sex: F1, 152 = 0.05, p = 0.95; Fig. 1).

Figure 1. Mean and standard deviation of individual HL in relation to age and sex.



The search in the zebra finch putative genome showed that the applied markers were widespread throughout the passerine genome (Table 1) and that the locus Fhy 1-25 was located in the exon of a solute carrier organic anion transporter gene. All loci conformed to Hardy-Weinberg equilibrium and no pair of loci showed significant linkage disequilibrium after Bonferroni correction. Identity disequilibrium was low and did not significantly differ from zero ($g_2 = 0.0006$, sd = 0.0008, $p = 0.17$).

Juvenile and adult survival

A significant presence of transient or emigrant individuals was detected among the birds marked for the first time in our study area ($Z = 2.27$, $p < 0.05$). We recalculated the GOF suppressing the first encounter of each individual (global test $\chi 2 = 7.06$, df = 11, $p = 0.79$), and found scarce evidence of remaining heterogeneity ($Z = 1.45$, $p = 0.07$). As a consequence, we accounted for the presence of transients in our survival models (φ*(sex*t) φ(sex*t) p(sex*t)) by estimating the initial apparent survival rate (φ*(sex*t)) separately from the survival rate of previously marked individuals (φ(sex*t)) in subsequent intervals (Pradel et al. 1997). Then, we added time and sex constraints to the CJS model to identify the most parsimonious model. Suppressing the sex differences in all model parameters but the initial encounter probability always improved the model fit by one QAICc point or more (not shown). Further constraining led to the best ranked model with constant probability of initial survival, constant but sex-specific probability of recapture, and time dependency for the probability of subsequent encounters (QAICc = 741.28, deviance 723.07; Table 2, model 1). The probability (± standard error) of apparent initial survival was 0.216 ± 0.031, and 0.536 ± 0.047 for the subsequent occasions, respectively. The encounter probabilities (± standard error) were 0.267 ± 0.070 and 0.437 ± 0.086 for males and females captured for the first time, and 0.725 ± 0.132, 0.636 ± 0.080, 0.945 ± 0.068, and 0.908 ± 0.111 for individuals in the following capture occasions, respectively.

Table 1. Characteristics of the microsatellite loci used in the study. Number of alleles (A), Heterozygosity observed (Ho) and expected (He) and chromosome location according to their position in the Zebra finch genome are shown.

Locus	A	Ho	He	Chromosome: Start (Pb)	ID nearest gen*	Distance (Pb)
f1-25	7	0.738	0.7554	20 / 10.407.082	07700	exon
f3-60	35	0.9543	0.961	9 / 14.479.023	09067	27.807
Fhy 216	8	0.518	0.521	1a / 63.001.644	11914	11.2742
Fhy 236	25	0.896	0.87	20 / 13.791.266	08550	11.5271
Fhy 237	6	0.399	0.392	3 / 7.930.679	02614	15.700
Fhy 301	14	0.856	0.884	2 / 92.250.591	07400	150.859
Fhy 304	10	0.79	0.803	4_random / 2.365.290	15203	763.363
Fhy 310	13	0.872	0.864	2 / 92.250.591	15081	2.257
Fhy 329	8	0.682	0.672	3 / 49.130.923	10789	68.805
Fhy 339	12	0.831	0.83	1 / 95.843.488	13407	3.561
Fhy 356	12	0.833	0.856	1a / 6.627.591	02324	29.231
Fhy 361	7	0.549	0.518	2 / 29.361.136	01714	195.077
Fhy 401	13	0.795	0.788	Un / 52.369.084	06481	696.145
Fhy 444	14	0.8757	0.8816	1 / 12.170.793	07097	293.505
Fhy 466	12	0.8362	0.8438	7 / 21.099.737	10012	5.274

* Last digits of the gene's ID in ENSEMBL. Prefix: ENSTGUG000000.

We then fitted all possible combinations of each individual covariate to the survival parameters to test for additional improvement of our basic candidate model (Table 2). Multilocus heterozygosity did not influence individual survival (model 10), nor it did differentially affect age classes (models 2 and 3 for juvenile and adult survival, respectively). The lack of association between MLH and survival remained when we included the interactions between MLH and phenotypic traits as body mass (models 14 and 13) or tarsus length (models 16 and 15). Likewise, the joint effect of MLH with potential stress factors like brood status (models 11 and 12 for the effects

of all brood status categories on juvenile and adult survival, respectively; see supplementary file for models with effects of MLH on each brood status category), brood size (models 5 and 6), hatching date (models 8 and 4), and the ectoparasite load (mites: models 17 and 18; blowflies: models 20 and 19) did not improve the basic model. Nevertheless, these variables by themselves had no effect on survival in these particular study years (see Supplementary file). Survival of individuals with either high or low MLH values could differ from those with intermediate MLH values, but the inclusion of the quadratic effect of heterozygosity was not supported by the data (models 9 and 7). Likewise, the inclusion of fledgling type (extra pair vs within pair; see Supplementary file for single effects and models 26 and 25 for its interaction with MLH), and both maternal (models 21 and 22) and paternal MLH (models 23 and 24) as well as their interaction with other variables did not improve the basic model (see Supplementary file).

We ran a model including all values of heterozygosity at single loci (i.e. 15 parameters) to test whether local effects were influencing either juvenile or adult survival. However, the QAICc of this model increased by more than 45 points compared to the best survival model, most likely due to the significant increase in the number of parameters. The absence of single loci effects on survival was confirmed with F-ratio tests (Szulkin et al. 2010). The variance explained did not differ between a model including heterozygosity at all single loci and a model including MLH for juvenile ($F_{27, 506} = 1.20$, $p = 0.22$) and adult ($F_{27, 506} = 0.94$, $p = 0.54$) ages.

DISCUSSION

Using a wild population of a migratory songbird we explored the relationship between MLH and survival at different life stages, as well as the possibility that potential HFCs were subject to environmental and/or breeding conditions. Contrary to our expectations, MLH was not related to survival either in the juvenile or in

Table 2. Main candidate models testing individual survival in relation to MLH combined with following variables: sex, tarsus length, body mass, brood status, brood size (NP), standardized hatching date (SdHD) and abundance of mites and blowflies (CA). HL_male and HL_female indicate fledgling MLH separate by sex whereas HL_mat and HL_pat indicate MLH of the genetic mother and father, respectively. Due to different samples sizes models cannot be compared together. For all models see the supplementary file.

No	Model	Delta QAICc	Num. Par	QDeviance	beta±SE simple	beta±SE interaction
	N=854					
1	{phi*(.) phi(.) p*(.*sex) p(t)}	0	9	723.07	**----**	**----**
2	{phi*(.) phi(.*sex*HL) p*(.*sex) p(t)}	1.55	12	718.49	0.18±0.24	-0.63±0.37
3	{phi*(.*sex*HL) phi(.) p*(.*sex) p(t)}	3.13	12	720.07	0.14±0.21	-0.40±0.30
4	{phi*(.) phi(.*SdHD*HL) p*(.*sex) p(t)}	4.31	12	721.25	-0.05±0.17	0.28±0.24
5	{phi*(.*NP*HL) phi(.) p*(.*sex) p(t)}	4.53	12	721.47	-0.03±0.15	0.05±0.16
6	{phi*(.) phi(.*NP*HL) p*(.*sex) p(t)}	4.99	12	721.93	-0.11±0.17	0.01±0.18
7	{phi*(.) phi(.*sex*HL^2) p*(.*sex) p(t)}	5.14	14	717.98	0.16±0.22	0.13±0.31
8	{phi*(.*SdHD*HL) phi(.) p*(.*sex) p(t)}	5.43	12	722.37	-0.04±0.15	0.13±0.18
9	{phi*(.*sex*HL^2) phi(.) p*(.*sex) p(t)}	6.30	14	719.13	-0.13±0.17	0.02±0.26
10	{phi*(.*sex*HL) phi(.*sex*HL) p*(.*sex) p(t)}	6.94	15	717.72	-0.39±0.29; 0.14±0.35	0.28±0.45; -0.04±0.39
11	{phi*(.) phi(.*brood status*HL) p*(.*sex) p(t)}	11.93	16	720.65	-0.94±1.62	
12	{phi*(.*brood status*HL) phi(.) p*(.*sex) p(t)}	13.38	16	722.10	0.42±0.87	

Table 2. Continued.

No	Model	Delta QAICc	Num. Par	QDeviance	beta±SE simple	beta±SE interaction
	N=834					
1	phi*(.) phi(.) p*(.*sex) p(t)	0	9	700.68	----	----
13	{phi*(.) phi(.*mass*HL) p*(.*sex) p(t)}	4.95	12	699.50	-0.09±0.17	-0.16±0.18
14	{phi*(.*mass*HL) phi(.) p*(.*sex) p(t)}	5.85	12	700.40	-0.02±0.15	0.01±0.15
	N=838					
1	phi*(.) phi(.) p*(.*sex) p(t)	0	9	705.18	----	----
15	{phi*(.) phi(.*tarsus*HL) p*(.*sex) p(t)}	5.23	12	704.29	-0.08±0.17	-0.12±0.17
16	{phi*(.* tarsus *HL) phi(.) p*(.*sex) p(t)}	5.96	12	705.02	0.03±0.15	0.02±0.15
	N= 782					
1	phi*(.) phi(.) p*(.*sex) p(t)	0	9	589.22	----	----
17	{phi*(.*mites*HL) phi(.) p*(.*sex) p(t)}	4.97	12	588.05	-0.08±0.16	0.02±0.25
18	{phi*(.) phi(.*mites*HL) p*(.*sex) p(t)}	5.72	12	588.79	-0.10±0.20	0.10±0.35
	N= 834					
1	phi*(.) phi(.) p*(.*sex) p(t)	0	9	706.73	----	----
19	{phi*(.) phi(.*CA*HL) p*(.*sex) p(t)}	4.87	12	705.48	-0.04±0.18	-0.09±0.18
20	{phi*(.*CA*HL) phi(.) p*(.*sex) p(t)}	5.21	12	705.82	0.02±0.15	-0.11±0.15

Table 2. Continued.

No	Model	Delta QAICc	Num. Par	QDeviance	beta±SE simple	beta±SE interaction
	N= 821					
1	phi*(.) phi(.) p*(.*sex) p(t)	0	9	693.58	**----**	**----**
21	{phi*(.*sex*HLmat) phi(.) p*(.*sex) p(t)}	2.28	12	689.73	-0.06±0.21	0.41±0.30
22	{phi*(.) phi(.*sex*HLmat) p*(.*sex) p(t)}	4.92	12	692.37	-0.23±0.23	0.23±0.31
	N= 821					
1	{phi*(.*HLpat) phi(.) p*(.*sex) p(t)}	0	9	629.73	**----**	**----**
23	{phi*(.*sex*HLpat) phi(.) p*(.*sex) p(t)	4.94	12	628.53	-0.30±0.24	0.08±0.33
24	{phi*(.) phi(.*sex*HLpat) p*(.*sex) p(t)	5.42	11	631.06	-0.16±0.28	0.28±0.37
	N= 823					
1	{phi*(.) phi(.) p*(.*sex) p(t)}	0	9	684.99	**----**	**----**
25	{phi*(.) phi(.*EPP*HL) p*(.*sex) p(t)}	5.51	12	684.37	0.11±0.20	0.21±0.39
26	{phi*(.*EPP*HL) phi(.) p*(.*sex) p(t)}	6.07	12	684.93	-0.04±0.17	0.03±0.38

subsequent adult life stages. Moreover, neither mean heterozygosity nor its variability changed with age as expected if heterozygosity at the measured loci was related to survival and only the fittest individuals were those surviving (David 1998; Cohas et al. 2009). The relationship between genetic diversity and fitness has been suggested to be sensitive to stressful environmental conditions (review in Keller and Waller 2002; Armbruster and Reed 2005), but the absence of HSC found here was consistent across seasons and independent of factors potentially affecting survival such as hatching date, brood status or numbers of ectoparasites in the nests. Further, survival was not influenced by the MLH of genetic parents. These results support the idea that the lack of HFCs was not context dependent or, at least, not sensitive enough for the influence of stressful conditions on HFC to be detected.

HFCs have been reported for many behavioral (Marshall et al. 2003; Tiira et al. 2003) and morphological (Ryder et al. 2010) traits, but these correlations should be strengthened when the analyzed traits are more directly related to fitness like fecundity, survival or lifetime reproductive success. These traits are affected by many loci, which favor the expression of deleterious recessive mutations and, thus, HFCs (Houle et al. 1996, Szulkin et al. 2010). Accordingly, positive relationships between heterozygosity and survival have been commonly reported in a variety of taxa (amphibians: Lesbarrères et al. 2005; mammals: Coulson et al. 1998, Acevedo-Whitehouse et al. 2006; birds: Hansson et al. 2001, 2004, Jensen et al. 2007). Most of these studies have explored survival in early life stages (i.e. embryo, juvenile or up to recruitment) likely due to the difficulties of obtaining repeated data across the lifespan of individuals in open populations, and also because the magnitude of HFC is expected to decrease with age (David 1998; Rijks et al. 2008; Cohas et al. 2009). If selection eliminates unfit individuals from the population, both the variability in individual heterozygosity and the likelihood of detecting HFCs should decrease with increasing age. However, evidence of HFCs with increasing age has been reported as well, with old individuals being more sensitive to environmental variation than young

ones (Charlesworth and Hughes 1996). In addition, even opposite effects of heterozygosity have been detected at different life stages with negative and positive effects in early and late life, respectively (Hardenberg et al. 2007; Escobar et al. 2008; Olano-Marin et al. 2011a,b). The lack of a relationship between MLH and the survival probability across all life stages in our population of pied flycatchers adds to inconsistencies in the literature in this type of study and highlights the need of additional work concerning HSC beyond juvenile stages to clarify the impact of inbreeding in open populations.

Variation in survival due to heterozygosity is likely to arise more (or only) under adverse conditions (Richardson et al. 2004; Armbruster and Reed 2005). An individual's ability to cope with stressful conditions may be determined by its genetic diversity, and will therefore vary among individuals. This may be due to either the increased likelihood of expression of deleterious alleles under certain environments or to highly heterozygous individuals being more likely than low heterozygous individuals to possess the allelic diversity needed to face adverse environmental conditions (Keller and Waller 2002; Armbruster and Reed 2005). In fact, evidence on context dependence in HFCs has been reported, e.g. for the Seychelles warbler, where HFCs occurred exclusively during low quality seasons (Richardson et al. 2004, Brouwer et al. 2007) or for the common frog (Rana temporaria), with stronger HFCs under restricted food environments (Lesbarrères et al. 2005). In the pied flycatcher, late hatching dates (Lundberg and Alatalo 1992), mating status (as secondary females usually do not receive male assistance in chick feeding; Lundberg and Alatalo 1992) or the abundance of nest ectoparasites (reducing nestling growth and survival prospects; Merino and Potti 1995) are factors potentially affecting fledgling survival. The absence of HFCs was stable across the stressors we explored, suggesting that the genetic diversity measured at this set of loci did not provide (enough) advantages to counteract the effects of the factors affecting survival. However, these variables had no effect on individual survival, even when the possibility of an interaction with

individual heterozygosity was ignored. This suggests that the environmental conditions during both study years were not harsh enough to produce sufficient variation in survival prospects related to the factors we explored. Additionally, we also explored the possibility that the heterozygosity of the genetic parents (as a specific parental effect; see e.g. Price 1998) could affect individual survival of pied flycatchers. Parental MLH is positively related to recruitment rate in blue tits (Cyanistes caeruleus; Olano-Marin et al. 2011) whereas in Seychelles warblers fledgling survival is influenced by the heterozygosity of both the genetic father (Richardson et al. 2004, but see Brouwer et al. 2007) and mother (Brouwer et al. 2007). Nevertheless, we failed to detect any relationship between individual survival and the heterozygosity of genetic parents. Likewise, neither the fledgling type (extra pair versus within pair young) nor the interaction between fledgling type and heterozygosity did influence individual survival.

The lack of a correlation between individual survival and heterozygosity found here may be due several reasons. First, because HFCs explain on average 1% of fitness variation (Chapman et al. 2009) and fledgling recruitment is low (on average, 12% in the study years), stochastic factors operating soon after fledging and/or during migration (e.g. severe adverse conditions and/or high rates of predation) may override any effect of heterozygosity on individual survival. A second, non-mutually exclusive possibility is that the lack of HFC may be consequence of strong selection pressure occurring in life stages previous to those analyzed here. Indeed, genetic diversity is known to be related with embryo and nestling survival (Keller and Waller 2002; Blomqvist et al. 2010). We cannot reject this possibility, but note that the largest differences in survival in our population during 2005 and 2006 occur after fledging, with a low proportion of both unhatched eggs (6.6 %, total eggs = 1333) and individuals dying in the nest (3.8 %, total individuals = 1205) in comparison to that of presumably dead, non-recruiting fledglings (86 %, total fledglings = 863). Third, we cannot discard a lack of statistical power to detect an HFC given that 600 individuals

would be needed to reach 80% of detection power (Coltman and Stale 2003) with an effect size between MLH and survival of r = 0.1 (the average value found by Coltman and Stale 2003). Our data set included between 782 and 854 individuals depending on the traits measured, but a large inbreeding variance in the population is also needed for HFCs to occur (Slate et al. 2004). Pedigree relationships based on 25 years of study show that matings between relatives are rare events in our population (1.2%; D. Canal and J. Potti, manuscript in preparation), suggesting a low inbreeding variance. This estimate, however, must be taken with some caution since the origin of many breeding individuals (53%) is unknown, and the number of inbred individuals may thus be underestimated. Furthermore, immigration may generate linkage disequilibrium which, together with random mating, produces identity disequilibrium (i.e. inbreeding; Szulkin et al. 2010). Finally, MLH could reflect uniquely heterozygosity at these loci. None of the loci appear to be linked to functional genes influencing survival because a model with all single loci did not better explain individual variation in survival than a model including MLH. Furthermore, as suggested by the lack of identity disequilibrium (estimated as g_2), the typed loci also seem to be unrelated to inbreeding at the genome level. HFCs, however, can occur even in the absence of significant g_2 due to wide inbreeding effects: given that traits are (usually) influenced by many more loci than those typed, inbreeding effects are more easily detected through HFC than through correlations in the loci (Szulkin et al. 2010). The number of markers needed to reflect general inbreeding values has been extensively discussed, and our panel of markers would not escape this debate. Although a larger panel of markers should apparently report more precise estimates of inbreeding (Slate et al. 2004), this argument should not be used to invalidate HFC work (Szulkin et al. 2010). In fact, a recent study of a zebra finch population (with low inbreeding variance) has challenged this view by showing that a panel of 11 microsatellites (the mean microsatellite number used in HFC studies; Chapman et al. 2009) located across the genome was as

informative as a panel of 1359 SNP markers or a 5th generation pedigree (Forstmeier et al. 2012).

A decade ago Keller and Waller (2002) highlighted the need of studies exploring the interaction between genetics, environment and fitness. Today, these types of studies are still very scarce. Recent work has highlighted the role of heterozygosity on fitness even in large natural populations with apparent absence of inbreeding (Wetzel et al. 2011), which could be determined by temporal (Harrison et al. 2011) and environmental conditions (Brouwer et al. 2007). Hence, we emphasize that studies exploring HFCs in populations with different demographic histories and under variable environmental conditions are required to increase our knowledge on the causes of HFCs.

SUPLEMENTARY FILE 1

Table 1. Candidate models testing for the effect of individual MLH combined with following variables: sex, study plot, tarsus length, body mass, brood status, brood size (NP), standardized hatching date (sdHD) and abundance of ectoparasites (mites and blowflies (Cal)). HL_male and HL_female indicate fledgling MLH separate by sex whereas HL_mat and HL_pat indicate MLH of the genetic mother and father, respectively. Sample size varies from the initial 863 individuals because the information of some variables was not available for all fledglings.

Model (N = 854)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	741.24	0.00	9	723.07	null	null
{phi*(.) phi(.*sex*HL_male) p*(.*sex) p(t)}	741.31	0.07	11	719.05	phi	sex*HL_male
{phi*(.*sex) phi(.) p*(.*sex) p(t)}	742.27	1.04	10	722.06	phi	sex
{phi*(.) phi(.*sex) p*(.*sex) p(t)}	742.76	1.52	10	722.55	phi	sex
{phi*(.*sex*HL_male) phi(.) p*(.*sex) p(t)}	742.81	1.57	11	720.56	phi*	sex*HL_male
{phi*(.) phi(.*sex*HL^2_male) p*(.*sex) p(t)}	743.34	2.10	12	719.04	phi	sex*HL^2_male
{phi*(.*sex*HL_female) phi(.) p*(.*sex) p(t)}	743.83	2.59	11	721.57	phi*	sex*HL_female
{phi*(.) phi(.*sex*HL_female) p*(.*sex) p(t)}	744.23	3.00	11	721.98	phi	sex*HL_female
{phi*(.*sex) phi(.*sex) p*(.*sex) p(t)}	744.24	3.00	11	721.99	phi*+phi	Sex
{phi*(.*sex*HL^2_male) phi(.) p*(.*sex) p(t)}	744.48	3.24	12	720.18	phi*	sex*HL^2_male
{phi*(.*HL) phi(.*HL) p*(.*sex) p(t)	744.98	3.74	11	722.72	phi*+phi	HL
{phi*(.*sex*HL^2_female) phi(.) p*(.*sex) p(t)}	745.31	4.08	12	721.02	phi*	sex*HL^2_female
{phi*(.) phi(.*sex*HL^2_female) p*(.*sex) p(t)}	745.80	4.56	12	721.50	phi	sex*HL^2_female

Table 1. continued

Model (N = 854)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.*sex*HL) phi(.) p*(.*sex) p(t)}	746.05	4.81	12	721.75	phi*	sex*HL
{phi*(.*NP) phi(.) p*(.*sex) p(t)}	741.82	0.58	10	721.61	phi*	broodsize
{phi*(.*NP) phi(.*NP) p*(.*sex) p(t)}	742.01	0.77	11	719.75	phi*+phi	broodsize
{phi*(.) phi(.*NP) p*(.*sex) p(t)}	742.54	1.30	10	722.33	phi	broodsize
{phi*(.*NP*HL) phi(.*NP*HL) p*(.*sex) p(t)}	749.73	8.49	15	719.27	phi*+phi	broodsize*HL
{phi*(.) phi(.*broodstatus) p*(.*sex) p(t)}	746.27	5.04	12	721.98	phi	broodstatus
{phi*(.*broodstatus) phi(.) p*(.*sex) p(t)}	747.15	5.91	12	722.85	phi*	broodstatus
{phi*(.*broodstatus) phi(.*broodstatus) p*(.*sex) p(t)}	752.06	10.82	15	721.60	phi*+phi	broodstatus
{phi*(.) phi(.*broodstatus*HL) p*(.*sex) p(t)}	753.169	11.93	16	720.65	phi	broodstatus*HL
{phi*(.*broodstatus*HL) phi(.) p*(.*sex) p(t)}	754.622	13.38	16	722.10	phi*	broodstatus*HL
{phi*(.*broodstatus*HL) phi(.*broodstatus*HL) p*(.*sex) p(t)}	766.00	24.76	23	718.93	phi*+phi	broodstatus*HL
{phi*(.*SdHD) phi(.) p*(.*sex) p(t)}	743.22	1.99	10	723.01	phi*	laying date
{phi*(.) phi(.*SdHD) p*(.*sex) p(t)}	743.27	2.04	10	723.06	phi	laying date
{phi*(.*SdHD^2) phi(.) p*(.*sex) p(t)}	744.94	3.71	11	722.69	phi*	laying date^2
{phi*(.) phi(.*SdHD^2) p*(.*sex) p(t)}	745.24	4.00	11	722.99	phi	laying date^2
{phi*(.*SdHD) phi(.*SdHD) p*(.*sex) p(t)}	745.26	4.02	11	723.01	phi*+phi	laying date
{phi*(.*SdHD^2*HL) phi(.) p*(.*sex) p(t)}	747.33	6.09	13	720.98	phi*	laying date^2*HL
{phi*(.) phi(.*SdHD^2*HL) p*(.*sex) p(t)}	748.72	7.48	13	722.37	phi	laying date^2*HL

Table 1. continued

Model (N = 854)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.*SdHD^2) phi(.*SdHD^2) p*(.*sex) p(t)}	749.03	7.79	13	722.68	phi*+phi	laying date^2
{phi*(.*SdHD*HL) phi(.*SdHD*HL) p*(.*sex) p(t)}	751.63	10.39	15	721.17	phi*+phi	laying date*HL
{phi*(.*SdHD^2*HL) phi(.*SdHD^2*HL) p*(.*sex) p(t)}	754.98	13.75	17	720.40	phi*+phi	laying date^2*HL
{phi*(.) phi(.*plot) p*(.*sex) p(t)}	742.18	0.95	10	721.97	phi	plot
{phi*(.*plot) phi(.) p*(.*sex) p(t)}	742.95	1.71	10	722.74	phi*	plot
{phi*(.*plot) phi(.*plot) p*(.*sex) p(t)}	743.21	1.98	11	720.96	phi*+phi	plot
{phi*(.) phi(.*plot*HL_plot1) p*(.*sex) p(t)}	743.97	2.73	11	721.71	phi	plot*HL_plot1
{phi*(.) phi(.*plot*HL_plot2) p*(.*sex) p(t)}	744.22	2.99	11	721.97	phi	plot*HL_plot2
{phi*(.*plot*HL_plot1) phi(.) p*(.*sex) p(t)}	744.27	3.03	11	722.02	phi*	plot*HL_plot1
{phi*(.*plot*HL_plot2) phi(.) p*(.*sex) p(t)}	744.58	3.34	11	722.33	phi*	plot*HL_plot2
{phi*(.*plot*HL) phi(.) p*(.*sex) p(t)}	745.90	4.66	12	721.60	phi*	plot*HL
{phi*(.) phi(.*plot*HL) p*(.*sex) p(t)}	746.01	4.77	12	721.71	phi	plot*HL

Table 2. Candidate models testing for the effect of HL combined with body mass on individual survival.

Model (N = 834)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	718.86	0.00	9	700.69	null	null
{phi*(.*mass) phi(.) p*(.*sex) p(t)}	720.64	1.78	10	700.42	phi*	body mass
{phi*(.) phi(.*mass) p*(.*sex) p(t)}	720.89	2.03	10	700.68	phi	body mass
{phi*(.*mass^2) phi(.) p*(.*sex) p(t)}	722.61	3.75	11	700.35	phi*	body mass^2
{phi*(.) phi(.*mass^2) p*(.*sex) p(t)}	722.62	3.76	11	700.36	phi	body mass^2
{phi*(.*mass) phi(.*mass) p*(.*sex) p(t)}	722.68	3.81	11	700.42	phi*+phi	body mass
{phi*(.*mass^2) phi(.*mass^2) p*(.*sex) p(t)}	726.31	7.45	13	699.95	phi*+phi	body mass^2
{phi*(.) phi(.*mass^2*HL) p*(.*sex) p(t)}	726.35	7.49	13	699.99	phi	body mass^2*HL
{phi*(.*mass^2*HL) phi(.) p*(.*sex) p(t)}	726.63	7.76	13	700.27	phi*	body mass^2*HL
{phi*(.*mass*HL) phi(.*mass*HL) p*(.*sex) p(t)}	729.51	10.65	15	699.04	phi*+phi	body mass*HL
{phi*(.*mass^2*HL) phi(.*mass^2*HL) p*(.*sex) p(t)}	734.06	15.20	17	699.45	phi*+phi	body mass^2*HL

Table 3. Candidate models testing for the effect of HL combined with tarsus length on individual survival.

Model (N = 838)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	723.37	0.00	9	705.19	null	null
{phi*(.*tarsus) phi(.) p*(.*sex) p(t)}	725.30	1.93	10	705.08	phi*	tarsus length
{phi*(.) phi(.*tarsus) p*(.*sex) p(t)}	725.35	1.98	10	705.13	phi	tarsus length
{phi*(.) phi(.*tarsus^2) p*(.*sex) p(t)}	726.75	3.39	11	704.49	phi	tarsus length^2
{phi*(.*tarsus^2) phi(.) p*(.*sex) p(t)}	727.21	3.84	11	704.95	phi*	tarsus length^2
{phi*(.*tarsus) phi(.*tarsus) p*(.*sex) p(t)}	727.32	3.96	11	705.07	phi*+phi	tarsus length
{phi*(.*tarsus^2) phi(.*tarsus^2) p*(.*sex) p(t)}	730.14	6.78	13	703.78	phi*+phi	tarsus length^2
{phi*(.) phi(.*tarsus^2*HL) p*(.*sex) p(t)}	730.46	7.09	13	704.10	phi	tarsus length^2*HL
{phi*(.*tarsus^2*HL) phi(.) p*(.*sex) p(t)}	731.25	7.89	13	704.90	phi*	tarsus length^2*HL
{phi*(.*tarsus*HL) phi(.*tarsus*HL) p*(.*sex) p(t)}	734.68	11.31	15	704.20	phi*+phi	tarsus length*HL
{phi*(.*tarsus^2*HL) phi(.*tarsus^2*HL) p*(.*sex) p(t)}	737.95	14.58	17	703.34	phi*+phi	tarsus length^2*HL

Table 4. Candidate models testing for the effect of sex combined with maternal HL on individual survival.

Model (N = 821)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	711.77	0.00	9	693.59	null	null
{phi*(.*sex*HLmatMale) phi(.) p*(.*sex) p(t)}	712.09	0.33	11	689.83	phi*	sex*HLmat_male
{phi*(.*sex) phi(.) p*(.*sex) p(t)}	712.72	0.95	10	692.50	phi*	sex
{phi*(.*HLmat) phi(.) p*(.*sex) p(t)}	713.10	1.33	10	692.88	phi*	HLmat
{phi*(.) phi(.*HLmat) p*(.*sex) p(t)}	713.24	1.47	10	693.02	phi	HLmat
{phi*(.) phi(.*sex) p*(.*sex) p(t)}	713.57	1.80	10	693.35	phi	sex
{phi*(.*HLmat) phi(.*HLmat) p*(.*sex) p(t)}	713.87	2.11	11	691.61	phi*+phi	HLmat
{phi*(.*sex*HLmatMale^2) phi(.) p*(.*sex) p(t)}	714.12	2.35	12	689.81	phi*	sex*Hlmat^2_male
{phi*(.) phi(.*sex*HLmatFemale) p*(.*sex) p(t)}	714.64	2.87	11	692.37	phi	sex*HLmat_female
{phi*(.*sex*HLmatFemale) phi(.) p*(.*sex) p(t)}	714.66	2.90	11	692.40	phi*	sex*HLmat_female
{phi*(.*sex) phi(.*sex) p*(.*sex) p(t)}	714.76	2.99	11	692.50	phi*+phi	sex
{phi*(.*HLmat^2) phi(.) p*(.*sex) p(t)}	715.06	3.29	11	692.80	phi*	HLmat^2
{phi*(.) phi(.*HLmat^2) p*(.*sex) p(t)}	715.28	3.51	11	693.02	phi	HLmat^2
{phi*(.) phi(.*sex*HLmatMale) p*(.*sex) p(t)}	715.61	3.84	11	693.35	phi	sex*HLmat_male
{phi*(.) phi(.*sex*HLmatFemale^2) p*(.*sex) p(t)}	716.37	4.60	12	692.06	phi	sex*HLmat^2_female
{phi*(.*sex*HLmatFemale^2) phi(.) p*(.*sex) p(t)}	716.71	4.94	12	692.40	phi*	sex*HLmat^2_female
{phi*(.) phi(.*sex*HLmatMale^2) p*(.*sex) p(t)}	717.64	5.87	12	693.33	phi	sex*Hlmat^2_male
{phi*(.*HLmat^2) phi(.*HLmat^2) p*(.*sex) p(t)}	717.87	6.10	13	691.51	phi*+phi	HLmat^2

Table 4. continued

Model (N = 821)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.*sex*HLmat) phi(.*sex*HLmat) p*(.*sex) p(t)}	718.66	6.89	15	688.18	phi*+phi	sex*HLmat
{phi*(.) phi(.*sex*HLmat^2) p*(.*sex) p(t)}	720.45	8.69	14	692.04	phi	sex*HLmat^2
{phi*(.*sex*HLmat^2) phi(.*sex*HLmat^2) p*(.*sex) p(t)}	726.34	14.57	19	687.57	phi*+phi	sex*HLmat^2

Table 5. Candidate models testing for the effect of sex combined with paternal HL on individual survival.

Model (N = 754)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.*HLpat) phi(.) p*(.*sex) p(t)}	647.92	0.00	9	629.73	phi*	HLpat
{phi*(.) phi(.) p*(.*sex) p(t)}	648.46	0.54	8	632.31	null	null
{phi*(.*HLpat^2) phi(.) p*(.*sex) p(t)}	649.62	1.69	10	629.38	phi*	HLpat^2
{phi*(.) phi(.*sex) p*(.*sex) p(t)}	649.85	1.93	9	631.65	phi	sex
{phi*(.) phi(.*HLpat) p*(.*sex) p(t)}	650.50	2.58	9	632.31	phi	HLpat
{phi*(.*sex) phi(.*sex) p*(.*sex) p(t)}	651.20	3.28	10	630.96	phi*+phi	sex
{phi*(.*sex) phi(.) p*(.*sex) p(t)}	651.30	3.38	10	631.06	phi*	sex
{phi*(.) phi(.*sex*HLpat_female) p*(.*sex) p(t)} correct}	651.57	3.64	10	631.33	phi	sex*HLpat_female
{phi*(.*HLpat) phi(.*HLpat) p*(.*sex) p(t)}	651.61	3.69	11	629.32	phi*+phi	HLpat
{phi*(.) phi(.*sex*HLpat_male) p*(.*sex) p(t)} correct}	651.62	3.70	10	631.38	phi	sex*HLpat_male
{phi*(.*sex*HLpat_female) phi(.) p*(.*sex) p(t)} correct}	651.73	3.80	11	629.44	phi*	sex*HLpat_female
{phi*(.) phi(.*HLpat^2) p*(.*sex) p(t)}	652.29	4.37	10	632.06	phi	HLpat^2
{phi*(.*sex*HLpat_male) phi(.) p*(.*sex) p(t)} correct}	652.44	4.51	11	630.15	phi*	sex*HLpat_male
{phi*(.*sex*HLpat^2_female) phi(.) p*(.*sex) p(t)} correct}	653.25	5.33	12	628.91	phi*	sex*HLpat^2_female
{phi*(.) phi(.*sex*HLpat^2_female) p*(.*sex) p(t)} correct}	653.32	5.40	11	631.03	phi	sex*HLpat^2_female
{phi*(.*sex*HLpat^2_male) phi(.) p*(.*sex) p(t)} correct}	654.46	6.54	12	630.12	phi*	sex*HLpat^2_male
{phi*(.) phi(.*sex*HLpat^2_male) p*(.*sex) p(t)} correct}	655.23	7.31	12	630.89	phi	sex*HLpat^2_male
{phi*(.*HLpat^2) phi(.*HLpat^2) p*(.*sex) p(t)}	655.35	7.42	13	628.95	phi*+phi	HLpat^2

Table 5. Continued

Model (N = 754)	QAICc	Delta QAICc	Num. Par	QDeviance	Constrained parameter	Additional variable
{phi*(.) phi(.*sex*HLpat^2) p*(.*sex) p(t)} correct}	656.67	8.75	13	630.28	phi	sex*HLpat^2
{phi*(.*sex*HLpat) phi(.*sex*HLpat) p*(.*sex) p(t)} correct}	657.69	9.77	15	627.17	phi*+phi	sex*HLpat
{phi*(.*sex*HLpat^2) phi(.*sex*HLpat^2) p*(.*sex) p(t)} correct}	664.77	16.85	19	625.94	phi*+phi	sex*HLpat^2

Table 6. Candidate models testing for the effect of sex combined with abundance of mites.

Model (N = 782)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	607.44	0.00	9	589.23	null	null
{phi*(.*mites) phi(.) p*(.*sex) p(t)}	608.56	1.13	10	588.31	phi*	mites abundance
{phi*(.*sex) phi(.) p*(.*sex) p(t)}	608.82	1.38	10	588.56	phi*	sex
{phi*(.) phi(.*sex) p*(.*sex) p(t)}	609.00	1.56	10	588.74	phi	sex
{phi*(.) phi(.*mites) p*(.*sex) p(t)}	609.39	1.95	10	589.13	phi	mites abundance
{phi*(.*sex* mites _female) phi(.) p*(.*sex) p(t)}	609.74	2.30	11	587.43	phi*	sex*mites abundance_female
{phi*(.* mites) phi(.*mites) p*(.*sex) p(t)}	609.90	2.47	11	587.60	phi*+phi	mites abundance
{phi*(.) phi(.*sex* mites _female) p*(.*sex) p(t)}	610.32	2.88	11	588.01	phi	sex*mites abundance_female
{phi*(.*sex) phi(.*sex) p*(.*sex) p(t)}	610.75	3.32	11	588.45	phi*+phi	sex
{phi*(.*sex* mites _male) phi(.) p*(.*sex) p(t)}	610.79	3.35	11	588.48	phi*	sex*mites abundance_male
{phi*(.) phi(.*sex* mites _male) p*(.*sex) p(t)}	610.93	3.50	11	588.63	phi	sex*mites abundance_male
{phi*(.*sex* mites) phi(.) p*(.*sex) p(t)}	611.72	4.28	12	587.36	phi*	sex*mites abundance
{phi*(.) phi(.*sex* mites *HL_male) p*(.*sex) p(t)}	611.84	4.40	13	585.41	phi	sex*mites abundance*HL_male
{phi*(.) phi(.*sex* mites) p*(.*sex) p(t)}	612.25	4.82	12	587.89	phi	sex*mites abundance
{phi*(.*sex* mites *HL_male) phi(.) p*(.*sex) p(t)}	613.22	5.78	13	586.80	phi*	sex*mites abundance*HL_male

Table 6. continued

Model (N = 782)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.*sex* mites *HL_female) phi(.) p*(.*sex) p(t)}	613.50	6.06	13	587.07	phi*	sex*mites abundance*HL_female
{phi*(.) phi(.*sex* mites *HL_female) p*(.*sex) p(t)}	613.64	6.20	13	587.21	phi	sex*mites abundance*HL_female
{phi*(.*sex* mites) phi(.*sex*MITES) p*(.*sex) p(t)}	616.18	8.74	15	585.62	phi*+phi	sex*mites abundance
{phi*(.* mites *HL) phi(.*mites *HL) p*(.*sex) p(t)}	617.61	10.17	15	587.05	phi*+phi	mites abundance*HL
{phi*(.*sex* mites *HL) phi(.*sex* mites *HL) p*(.*sex) p(t)}	628.59	21.15	23	581.28	phi*+phi	sex*mites abundance*HL

Table 7. Candidate models testing for the effect of sex combined with abundance of blowflies (*Calliphora sp.*).

Model (N = 834)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	724.92	0.00	9	706.74	null	null
{phi*(.*sex) phi(.) p*(.*sex) p(t)}	725.93	1.02	10	705.72	phi*	sex
{phi*(.) phi(.*CA) p*(.*sex) p(t)}	726.06	1.14	10	705.84	phi	Calliphora abundance
{phi*(.) phi(.*sex) p*(.*sex) p(t)}	726.29	1.37	10	706.07	phi	sex
{phi*(.*sex*CA_male) phi(.) p*(.*sex) p(t)}	726.61	1.69	11	704.35	phi*	sex*Calliphora abundance_male
{phi*(.*CA) phi(.) p*(.*sex) p(t)}	726.62	1.71	10	706.41	phi*	Calliphora abundance
{phi*(.) phi(.*sex*CA_female) p*(.*sex) p(t)}	727.50	2.58	11	705.24	phi	sex*Calliphora abundance_female
{phi*(.*sex*CA_female) phi(.) p*(.*sex) p(t)}	727.83	2.91	11	705.57	phi*	sex*Calliphora abundance_female
{phi*(.*sex) phi(.*sex) p*(.*sex) p(t)}	727.83	2.92	11	705.57	phi*+phi	sex
{phi*(.) phi(.*sex*CA_male) p*(.*sex) p(t)}	728.18	3.26	11	705.92	phi	sex*Calliphora abundance_male
{phi*(.*sex*CA*HL_male) phi(.) p*(.*sex) p(t)}	729.10	4.19	13	702.75	phi*	sex*Calliphora abundance*HL_male
{phi*(.) phi(.*sex*CA*HL_male) p*(.*sex) p(t)}	729.51	4.59	13	703.15	phi	sex*Calliphora abundance*HL_male
{phi*(.) phi(.*sex*CA*HL) p*(.*sex) p(t)}	730.27	5.35	14	701.86	phi	sex*Calliphora abundance*HL
{phi*(.) phi(.*sex*CA*HL_female) p*(.*sex) p(t)}	730.66	5.75	13	704.30	phi	sex*Calliphora abundance*HL_female
{phi*(.*CA*HL) phi(.*CA*HL) p*(.*sex) p(t)}	730.92	6.01	13	704.56	phi*+phi	Calliphora abundance*HL

Table 7. Continued

Model (N = 834)	QAICc	Delta QAICc	Num. Par	Q Deviance	Constrained parameter	Additional variable
{phi*(.*sex*CA*HL_female) phi(.) p*(.*sex) p(t)}	731.06	6.15	13	704.71	phi*	sex*Calliphora abundance*HL_female
{phi*(.*sex*CA*HL) phi(.) p*(.*sex) p(t)}	734.27	9.35	16	701.74	phi*	sex*Calliphora abundance*HL
{phi*(.*sex*CA*HL) phi(.*sex*CA*HL) p*(.*sex) p(t)}	737.10	12.18	19	698.35	phi*+phi	sex*Calliphora abundance*HL

Table 8. Candidate models testing for the effect of sex combined with the type of fledgling (extra pair vs within pair young).

Model (N = 823)	QAICc	Delta QAICc	Num. Par	QDeviance	Constrained parameter	Additional variable
{phi*(.) phi(.) p*(.*sex) p(t)}	703.175	0	9	684.9958	null	null
{phi*(.) phi(.*EPP) p*(.*sex) p(t)}	705.013	1.8384	10	684.7943	phi	EPP
{phi*(.*EPP) phi(.) p*(.*sex) p(t)}	705.214	2.0394	10	684.9952	phi*	EPP
{phi*(.*EPP) phi(.*EPP) p*(.*sex) p(t)}	707.02	3.8452	11	684.757	phi*+phi	EPP
{phi*(.*sex*EPP) phi(.) p*(.*sex) p(t)}	707.857	4.6824	12	683.5461	phi*	sex*EPP
{phi*(.) phi(.*sex*EPP) p*(.*sex) p(t)}	708.625	5.45	12	684.3137	phi	sex*EPP
{phi*(.*sex*EPP) phi(.*sex*EPP) p*(.*sex) p(t)}	713.627	10.4523	15	683.1473	phi*+phi	sex*EPP

CAPITULO VI

Towards the simplification of MHC typing protocols: targeting classical MHC class II genes in a passerine, the pied flycatcher *Ficedula hypoleuca*

Secuencias de MHC clase II de papamoscas cerrojillo

Canal D, Alcaide M, Anmarkrud JA, Potti J. 2010. Towards the simplification of MHC typing protocols: targeting classical MHC class II genes in a passerine, the pied flycatcher *Ficedula hypoleuca*. BMC Res Notes. 3:236.

http://www.biomedcentral.com/1756-0500/3/236/abstract

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ABSTRACT

Major Histocompatibility Complex (MHC) has drawn the attention of evolutionary biologists due to its importance in crucial biological processes, such as sexual selection and immune response in jawed vertebrates. However, the characterization of classical MHC genes subjected to the effects of natural selection still remains elusive in many vertebrate groups. Here, we have tested the suitability of flanking intron sequences to guide the selective exploration of classical MHC genes driving the co-evolutionary dynamics between pathogens and their passerine (Aves, Order Passeriformes) hosts. Taking the pied flycatcher *Ficedula hypoleuca* as an example, we demonstrate that careful primer design can evade non-classical MHC gene and pseudogene amplification. At least four polymorphic and expressed loci were co-replicated using a single pair of primers in five non-related individuals (N=28 alleles). The cross-amplification and preliminary inspection of similar MHC fragments in eight unrelated songbird taxa suggests that similar approaches can also be applied to other species.

Keywords: songbirds, pathogen-mediated selection, sexual selection, adaptive variation, locus-specific typing

INTRODUCTION

For the last two decades, the Major Histocompatibility Complex (MHC) has drawn the attention of evolutionary biologists due to its importance in crucial biological processes, such as sexual selection and immune response in jawed vertebrates (reviewed in Tregenza and Wedell 2000; Sommer 2005; Piertney and Oliver 2006). Classical MHC genes, unlike those classified as non-classical, usually display extensive levels of genetic variability and ubiquitous expression patterns (Klein 1987). Among classical MHC loci, most research has focused on the second and third exons of class I genes and the second exon of class II B genes because of their traditional consideration as primary targets of pathogen-mediated selection. These highly polymorphic exons encode the extracellular domains that bind and present foreign peptides (antigens) to specialised CD4+ and CD8+ lymphocytes. Subsequently, the recognition of the complex MHC molecule-foreign antigen by T-lymphocytes triggers adaptive immunity (Klein 1986).

The characterization of classical MHC genes subjected to the effects of natural selection still remains elusive in many vertebrate groups (Babik et al. 2009). MHC genes belong to an extremely dynamic multigene family characterized by frequent gene duplication and loss, presence of pseudogenes, gene conversion and chromosome reorganization (Nei et al 1997; Richman et al. 2003; Lambracht-Washington and Lindahl 2004; Miller and Lambert 2004; Yuhki et al. 2007). Such complex evolutionary patterns could account for the substantial variation reported in MHC architecture and genome organization between and within different vertebrate groups (Shiina et al. 2004; Kelley et al 2004; Yuhki et al. 2007; Mehta et al. 2009), and sometimes even within the same species (Bontrop et al. 2006; Ekblom et al. 2007). Like other multigene families, the MHC is thought to be the subject of both birth-and-death and concerted evolution, yet the distinction between the two evolutionary models is sometimes difficult and controversial (Nei and Rooney 2005). The birth-

and-death model implies the creation of new genes by gene duplication, some of them being functionally retained in the genome for long time periods whereas others become inactivated (pseudogenes) or deleted from the genome. The concerted evolution hypothesis predicts that MHC genes evolve as a unit, mainly because of repeated gene conversion events across different members of the gene family (Nei and Rooney 2005). The implications of different forms of multigene family evolution are nevertheless crucial for MHC genotyping.

A prominent role of the birth-and-death evolutionary model has been typically associated with the mammalian MHC. Due to the independent evolution of MHC genes during long periods, MHC alleles usually form clusters according to loci. Such clusters allow tracing of orthologous relationships within and between different mammalian lineages (Hughes and Nei 1988; Kumanovics et al. 2003). This phenomenon has indeed facilitated the design of locus-specific primers across different mammalian groups (e.g. Snibson et al. 1998; Bettinotti et al. 2003; Weber et al. 2004). Non-mammalian lineages, on the other hand, usually exhibit a lack of orthologous relationships even on short evolutionary time scales (Nei et al. 1997; Kumanovics et al. 2003). In those groups, MHC sequences commonly fail to cluster according to loci (Alcaide et al. 2007; Hauswaldt et al. 2007; Glaberman and Caccone 2008) and, consequently, the assignment of alleles to particular genes becomes challenging. This phenomenon has been mainly attributed to concerted evolution that manifests in the homogenization of DNA sequences among different loci (Hess and Edwards 2002; Nei and Rooney 2005). Therefore, high rates of concerted evolution hinder MHC typing protocols due to co-amplification of multiple loci (e.g. Ekblom et al. 2003; Alcaide et al. 2007) and increased risk of chimera formation during PCR amplification (Lenz and Becker 2008). Taxa exhibiting extraordinarily high numbers of gene duplications and pseudogenes, such as songbirds and some fish, may be especially problematic (Málaga-Trillo et al. 1998, Reusch et al. 2001; Westerdahl 2007; Anmarkrud et al. 2010; Bollmer et al. 2010).

Degenerate primers targeting conserved coding regions of exon 2 have proven successful for the isolation of MHC class II B sequences in non-model avian species (Edwards et al 1995; Tsuda et al. 2001; Ekblom et al. 2003; Alcaide et al. 2007). Particularly in passerines, degenerate primers are expected to target (multiple) classical MHC genes, non-classical MHC genes and even pseudogenes (e.g. Aguilar et al. 2006; Anmarkrud et al. 2010; Bollmer et al. 2010). In this respect, a focus on evolutionarily relevant loci is needed in these species to diminish both laboratory efforts and costs. Despite strong evidence of concerted evolution in the avian MHC (Wittzell et al. 1999; Alcaide et al 2007; Westerdahl 2007), a few studies in birds have demonstrated that comprehensive knowledge of gene structure can be critical for the design of locus-specific primers that amplify the entire coding sequence of the targeted exon 2 (Miller and Lambert 2004; Worley et al. 2008; Burri et al. 2008a; Burri et al. 2008b; Silva and Edwards 2009). In this study, we have applied a multi-step PCR approach to obtain genomic MHC sequences in passerines (including both introns and exons). Our main goal was to test the suitability of flanking intron sequences to assist the specific amplification of the entire coding sequence of exon 2 from classical MHC class II B genes in passerines, a particularly challenging group regarding MHC genes.

METHODS

Study Species

We used the pied flycatcher *Ficedula hypoleuca (Aves: Muscicapidae)* as a model species. We also used eight non-related species to get a preliminary glimpse about the suitability of our molecular approach across other songbird families. The selected species were the white wagtail *Motacilla alba (Motacillidae)*, the common raven *Corvus corax (Corvidae)*, the European robin *Erithacus rubecula (Muscicapidae)*, the woodchat shrike *Lanius senator (Laniidae)*, Dupont's lark *Chersophilus duponti (Alaudidae)*, the

Sardinian warbler *Sylvia melanocephala (Sylviidae)*, the trumpeter finch *Bucanetes githagineus (Fringillidae)* and the chiffchaff *Phylloscopus collibita (Phylloscopidae)*.

Table 1. List of primer sequences used and/or developed in this study for PCR and sequencing. Standard IUB codes are used for degenerate primers.

Primer Name	Sequence (5'-3')	Reference
326	GAGTGYCAYTAYYTNAAYGGYAC	Ekblom et al. (2003)
325	GTAGTTGTGNCKGCAGTANSTGTCCAC	Ekblom et al. (2003)
MHC05	CGTRCTGGTGGCACTGGTGGYGCT	Miller & Lambert (2004)
RapEx3CR	CAGGCTGRCGTGCTCCAC	Alcaide et al. (2007)
MHC-F1	GAGTGTYVCTTCATTAACGGCAC	Anmarkrud et al. 2010
MHC-R1	CKCGTAGTTGTGCCGGCA	Anmarkrud et al. 2010
MHCIIFihy-I1F	CCTGYACAAACAGRGKTKTTCC	This study
MHCIIFihy-I2R	GCTCTGCCCCACGCTCAC	This study
MHCIIFihy-pE2R	ACCTCACCTTCTCCGTGC	This study
MHCIIFihy-pE2F	AAYGGCACGGAGAAGGTG	This study
MHCIIFihy-lwE2F	CATTAAYGGCACCAGCCGG	This study
MHCIIFihy-psE2R	TCCTCTCCACCAACCTCACGCA	This study
MHCIIFihy-E2CF	CCGTGTCCTGCACACACAGC	This study
MHCIIFihy-E2CR	GGGACASGCTCTGCCCCG	This study
MHCIIPas-E2iF	GAGTGTYACTTCATTAACGGCAC	This study
MHCIIPas-E2iR	CYNGTAGTTGTGNCGGCAG	This study

DNA and RNA extraction

Genomic DNA from five unrelated pied flycatchers was extracted from blood samples using the E.Z.N.A Blood extraction kit (Omega Bio-Tek, GA, USA). We used the HotSHOT protocol (Truett et al. 2000) to obtain genomic DNA from ethanol-preserved blood samples from one specimen of each additional passerine

species mentioned above. To discern between expressed MHC genes and non-functional pseudogenes, total RNA was isolated from approximately 100 µl of fresh blood taken from one pied flycatcher individual using TRIzol® LS Reagent (Invitrogen, CA, USA) according to the supplier's protocol. About 1 µg of total RNA was treated with DNase I (Sigma-Aldrich, MO, USA) before being reverse transcribed with the iScript™ cDNA synthesis kit (BioRad, CA, USA) to control for the possible amplification of target loci from genomic DNA. The cDNA was subsequently used as template for PCR amplification (see below).

PCR Amplification of genomic MHC fragments in passerines

For each passerine species, we used two sets of primers targeting conserved regions of MHC class II B genes in birds: MHC05 (Miller and Lambert 2004) and 325 (Ekblom et al. 2003) amplified genomic fragments spanning exons 1 to 2, whereas using primers 326 (Ekblom et al. 2003) and RapEx3CR (Alcaide et al. 2007) a partial region of exon 2, the entire intron 2 and a stretch of exon 3 were amplified (Table 1 and Figure 1; steps 1 and 2). The logic behind this step was to examine the intron sequences flanking exon 2 among species and among loci. PCRs were carried out using a PTC-100 Programmable Thermal Controller (MJ Research) in a final volume of 30 µl containing 1 unit of a commercial Taq Polymerase (Bioline, London, UK), 1X manufacturer-supplied buffer (Bioline), 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 5% Dimethyl sulfoxide (DMSO), 10 µg of BSA (Bovine Serum Albumin - Amersham Biosciences, Uppsala, Sweden), 10 pmoles of each primer and 1 µl of DNA extracts. PCRs were performed according to a touch down protocol from 66 ºC to 50 ºC (N=16 cycles) plus 19 cycles of annealing temperatures at 50 ºC. Cycling programs consisted of a first denaturing cycle of 3 min at 94 ºC, plus subsequent steps of 94 ºC for 40s, annealing steps for 40s and extension steps at 72 ºC for 40s. PCR amplicons were cloned and sequenced as described below except in the case of the pied

flycatcher (see the next subsection for a detailed description of the methods used in this species).

Targeting classical MHC class II B genes in pied flycatchers

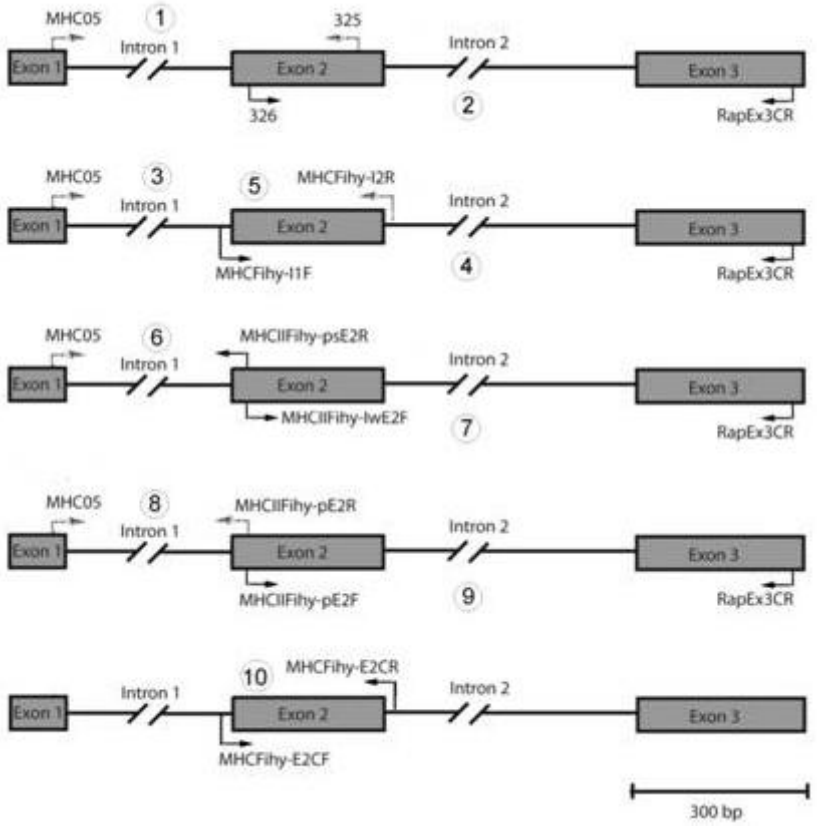
Genomic fragments spanning exons 1 to 2 and exons 2 to 3 (reactions 1 and 2, Figure 1) were directly sequenced (see methods below) using primers 325 and 326, respectively, in the case of the five pied flycatchers. Although direct sequencing chromatograms were mostly noisy, we were able to design new primers in conserved intron 1-exon 2 (MHCIIFihy-I1F) and exon 2-intron 2 junctions (MHCIIFihy-I2R, Table 1 and Figure 1). These nucleotide positions were among those of best quality across sequencing chromatograms and we failed to detect nucleotide polymorphisms within or between individuals. Primers MHCIIFihy-I1F and MHCIIFihy-I2R were used in combination with primers MHC05 and RapEx3CR to generate a pool of genomic fragments along the MHC class II domain (steps 3, 4 and 5, Figure 1). Primers MHC05 and MHCIIFihy-I2R (reaction 3, Figure 1) preferentially amplified an oligomorphic gene in the five individuals. Molecular cloning and sequencing (see below) revealed the occurrence of six different alleles and four non-synonymous nucleotide substitutions in exon 2 (GenBank Acc No. GU390299-GU390301). However, the examination of cDNA sequences (see below) confirmed that this locus was transcribed and may therefore represent a non-classical MHC gene. Primers MHCIIFihy-I1F and RapEx3CR (reaction 4, Figure 1), on the other hand, targeted at least one pseudogene as suggested by the occurrence of stop codons and frameshift mutations in the coding region of exon 2 (GenBank Acc. No. GU390297). Finally, direct sequencing of the PCR products obtained with the new primers MHCIIFihy-I1F and MHCIIFihy-I2R (reaction 5, Figure 1) denoted the co-amplification of non-classical MHC genes and pseudogenes along with highly polymorphic, classical MHC genes. We realized about the amplification of polymorphic MHC genes after

comparing the ambiguous nucleotide positions among the direct sequencing chromatograms obtained from different individuals.

In the following step, we tried to obtain intron sequences flanking exon 2 for each type of MHC loci (i.e. non-classical, classical and pseudogenes) using direct sequencing. To this aim, we profited from short nucleotide motifs within the sequence of exon 2 differing among loci (Figure 2A). This information was used to design primers for selective amplification and sequencing of specific intron sequences. Thus, primers MHCIIFihy-lwE2F (Table 1 and reaction 7, Figure 1) and RapEx3CR amplified intron 2 of non-classical MHC genes. Primers MHCIIFihy-pE2R and MHCIIFihy-pE2F (Table 1), in conjunction with primers MHC05 and RapEx3CR, amplified intron 1 and intron 2 sequences of classical MHC genes (reactions 8-9, Figure 1). We nonetheless failed to amplify intron 1 sequences from pseudogenes with primers MHCIIFihy-psE2R and MHC05 (Table 1 and reaction 6, Figure 1). This may result from the lack of this region in pseudogenes or due to the presence of extremely long introns difficult to amplify with our PCR protocol.

The alignment of flanking intron sequences showed differences in nucleotide composition suitable for the design of loci-specific primers (Figure 2A). Thus, in a last step we designed a set of new primers (MHCIIFihy-E2CF and MHCIIFihy-E2CR; Table 1 and reaction 10, Figure 1), aimed to specifically amplify classical MHC genes while overcoming the co-amplification of low polymorphic and pseudogenes. Direct sequencing revealed that highly polymorphic genes might share common flanking intron sequences (data not shown) and, therefore, the design of locus-specific primers was not feasible within this group.

Figure 1. Reactions carried out in this study to amplify MHC class II B sequences from genomic DNA in passerines.



Primer pairs used in each reaction:

1. MHC05 - 325

2. 326 - RapEx3CR

3. MHC05 - MHCIIFihy-I2R

4. MHCIIFihy-I1F - RapEx3CR

5. MHCIIFihy-I1F - MHCIIFihy-I2R

6. MHC05 - MHCIIFihy-psE2R

7. MHCIIFihy-lwE2F - RapEx3CR

8. MHC05 - MHCIIFihy-pE2R

9. MHCIIFihy-pE2F - RapEx3CR

10.MHCIIFihy-E2CF-MHCIIFihy-E2CR

PCR amplification of cDNA

cDNA was amplified in one pied flycatcher individual employing the MHC class II exon 2 specific primers pair, MHC-F1 and MHC-R1 (Anmarkrud et al. 2010, Table 1). The target of interest was amplified using the cDNA as template with similar PCR conditions and PCR purification approach as described in Anmarkrud et al. (2010).

Molecular cloning and sequence analyses

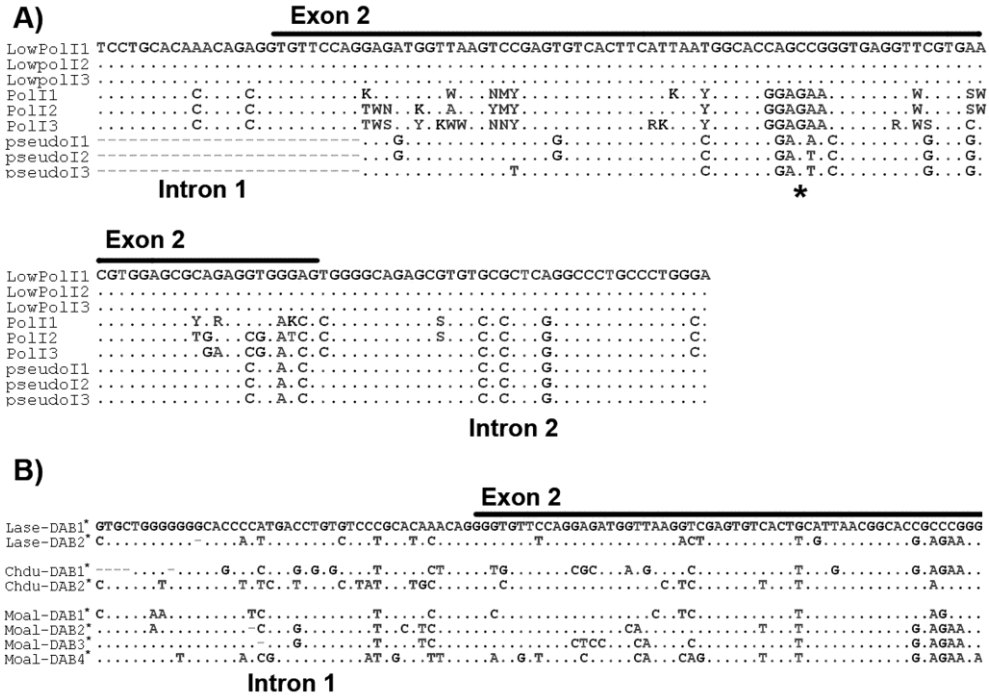
PCR amplicons from genomic DNA and cDNA were cleaned-up in Microcon centrifuge tubes (Millipore) and subsequently cloned into bacterial plasmids using the PGEM-T easy vector system II (Promega, WI, USA). MHC inserts from positive clones were amplified as described above using the vector specific M13 primers. The PCR products were visualized on 1.5 % agarose gels and inserts suspected to contain the target loci were sequenced using the BigDye 1.1 kit (Applied Biosystems, CA, USA). Between 8 and 16 positive clones per individual were analysed for PCR products obtained during the PCRs 1 to 4 (Figure 1). A total of 100 clones (20 per individual) were randomly screened for PCR products described in step 10 (Figure 1). Labelled fragments were resolved in an ABI3130xl automated sequencer (Applied Biosystems). MHC sequences were edited and aligned in BioEdit v. 7.0.9 (Hall 1999). The phylogenetic relationships among MHC sequences were visualized using Neighbor-net networks constructed in SplitsTree 4.0 (Huson et al. 2006) according to the Kimura-2-parameter model.

Tests for selection

An excess of non-synonymous (d_N) over synonymous (d_S) substitutions characterizes coding sequences under positive selection (Garrigan et al. 2003). Functional

constraints in protein structure and function are translated, on the contrary, into an excess of synonymous substitutions (i.e. stabilizing or purifying selection). To detect selective signatures, d_N/d_S rates were calculated in MEGA 4.1 (Kumar et al. 2008)

Figure 2. A. Nucleotide differences among low polymorphic, high polymorphic MHC class II genes and pseudogenes in the pied flycatcher. Sequences obtained from three different individuals are shown. Motifs allowing the design of loci-specific primers within exon 2 are pointed out with an asterisk. **B.** Nucleotide variations in the flanking introns of two presumably distinct MHC class II B genes of the woodchat shrike (Lase) and Dupont's Lark (Chde) along with four putatively distinct genes isolated in the white wagtail (Moal). Genes are labelled as DAB1*, DAB2* and so on. Asterisks indicate that the identity of these putative genes is not still confirmed, and future studies may confirm their identity.



using a Modified Nei-Gojobori method with Jukes-Cantor correction. Standard errors were calculated with 1,000 bootstrap replicates. Codons thought to be involved in antigen recognition were analysed independently from those presumably not involved in such function. We used information derived from the well-studied MHC class II molecule of humans (Brown et al. 1993) to delimitate putative antigen-binding regions. Statistical support for positive selection was evaluated through Z-tests run in MEGA 4.1.

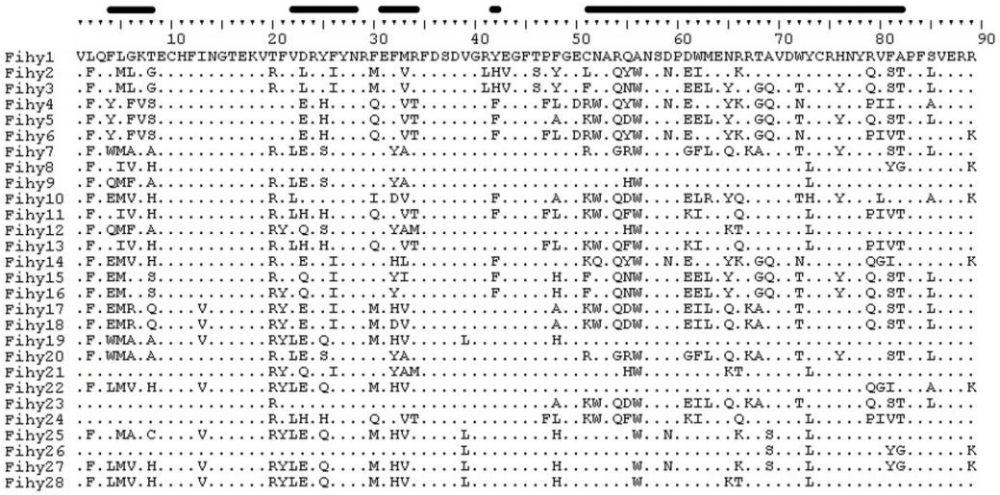
RESULTS

Genetic diversity and molecular evolution of classical MHC class II genes in pied flycatchers

We successfully and selectively amplified highly polymorphic, classical MHC class II genes in pied flycatchers using primers MHCIIFihy-E2CF and MHCIIFihy-E2CR (Table 1, step 10 in Figure 1). The analysis of 100 clones across the 5 individuals revealed 28 class II alleles translated into 28 amino acid sequences (GenBank Acc. No GU390232-GU390259, see Figure 3). For each individual, about 20 % of the cloned alleles suspiciously resembled chimeric sequences or base misincorporations during bacterial replication and were discarded. In this respect, allele similarity was much higher within individuals than among individuals. Positive clones interchanging the first 30 bp of the 5' end of exon 2 were abundant. This finding hints at strong competition during the completion of PCR amplicons and the use of incomplete PCR amplicons as templates for subsequent amplification steps. The removal of putatively false and spurious alleles from our data set revealed between 5 and 8 alleles per individual, a finding in agreement with co-amplification of minimum 4 classical MHC class II B loci in pied flycatchers. The analysis of 267 bp of the exon 2 revealed high genetic polymorphism, with a large number of segregating sites (S =112) resulting from 159 mutations, an average nucleotide diversity among sites (π = 0.167) and 44.63

nucleotide differences, on average, among alleles. For those codons located within putative antigen binding regions, non-synonymous substitutions were remarkably more frequent than synonymous substitutions (dN = 0.587 ± 0.084; dS = 0.190 ± 0.043; Z-test, P < 0.001). This was not the case for those codons not presumably interacting with antigens directly (dN= 0.054 ± 0.016; dS = 0.058 ± 0.021; Z-test, P = 0.79). Phylogenetic networks allow distinguishing a large cluster of sequences

Figure 3. Predicted amino acid sequences of 28 MHC class II alleles in the pied flycatcher. Dots indicate identity with the top sequence. Black bars indicate the main coding regions exhibiting strong positive selection in the human MHC class II molecule (Brown et al. 1993).



containing exon 2 sequences from classical MHC loci and a different cluster containing exon 2 sequences from non-classical MHC loci. Pseudogene sequences failed to intermingle with either of these two distinct clusters (Figure 4). A clustering of sequences according to loci is not evident within the exon 2 sequences derived from classical MHC genes. The phylogenetic network suggests the occurrence of

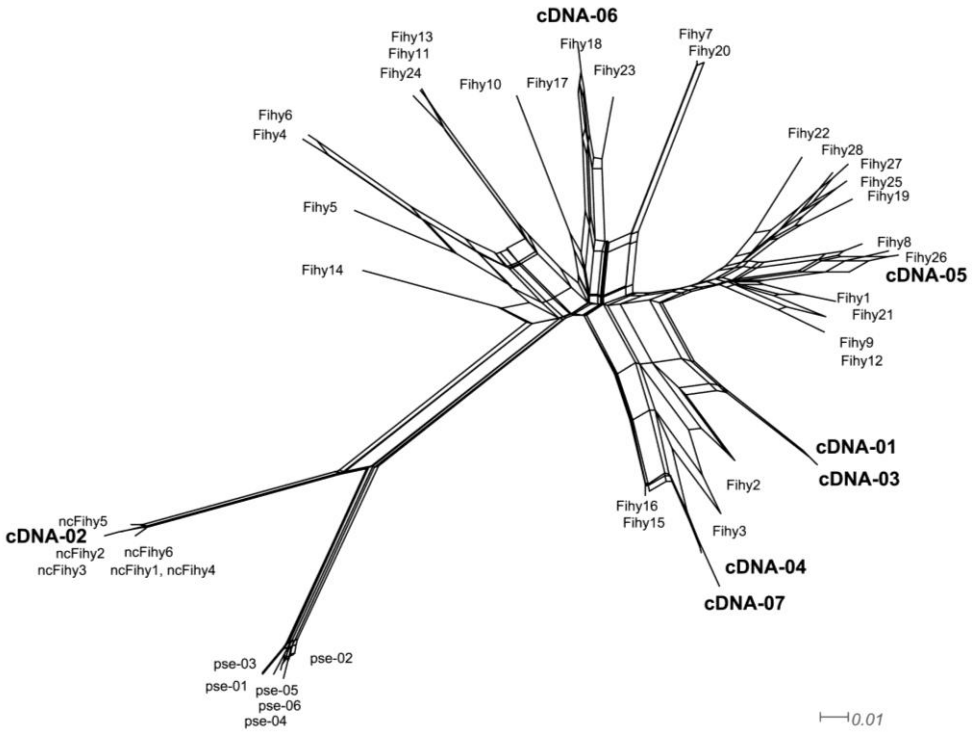
divergent and recombining allele lineages that are shared among different loci instead. Finally, we isolated up to seven different cDNA sequences from the same individual. These cDNA sequences intermingle with the two clusters representing classical and non-clasical genes. Some of the cDNA sequences obtained in the individual investigated were identical or related to the sequences isolated from genomic DNA in other individuals (see Figure 4). Thus, our cDNA sequence data confirmed that both classical and non-classical MHC class II genes were transcribed in blood.

Cross-amplification of genomic MHC fragments in passerines

Primers 326 and 325, in combination with MHC05 and RapEx3CR (equivalent to reaction 1 and 2, Figure 1) successfully amplified MHC class II genomic fragments across a wide variety of passerine species. Sequences from the white wagtail (GU390288-GU390293), common raven (GU390281-GU390283), European robin (GU390284), woodchat shrike (GU390281-GU390283), Dupont's lark (GU390277-GU390280), Sardinian warbler (GU390294-GU390296), trumpeter Finch (GU390273-GU390276) and chiffchaff (GU390293) were deposited in GenBank (see also Additional files 1 and 2). Our set of MHC sequences reported intron 1 sizes ranging from 299 to 478 bp and intron 2 sizes ranging from 190 to 350 bp in the species investigated. In those cases where we failed to obtain complete intron sequences from clones (especially in the case of intron 2), intron size was estimated through examination of 1.5% agarose gels. The alignment of intron sequences suggested the co-amplification of multiple copies in some species, such as the white wagtail, the woodchat shrike and Dupont's lark (Figure 2B). The phylogenetic network of intron 1 did not cluster according to species (Figure 5). This finding thus suggests that some regions of the multigene family can be gene conversion free and concerted evolution may not be ubiquitous throughout all the multigene family. For the chiffchaff, the European robin, the common raven, the trumpeter Finch or the Sardinian warbler,

our PCR experiments seemed to preferentially amplify particular MHC fragments (see Additional Files 1 and 2 for sequence data). However, the low number of clones analysed per species prevents this information being conclusive so far. All isolated sequences seemed to be putatively functional, as manifested by the lack of stop codons or frameshift mutations. However, not all the sequences obtained using both sets of primers were overlapping and we could not create contigs in all cases. As a result, more detailed examination for other species rather than the pied flycatcher is needed.

Figure 4. Neighbor-net network of exon 2 sequences isolated from classical (Fihy1-28), non-classical (ncFihy1-6) and at least one MHC class II B pseudogene (pse01-06) in five pied flycatchers. Seven cDNA sequences (cDNA01-07) isolated from a different individual are also shown.

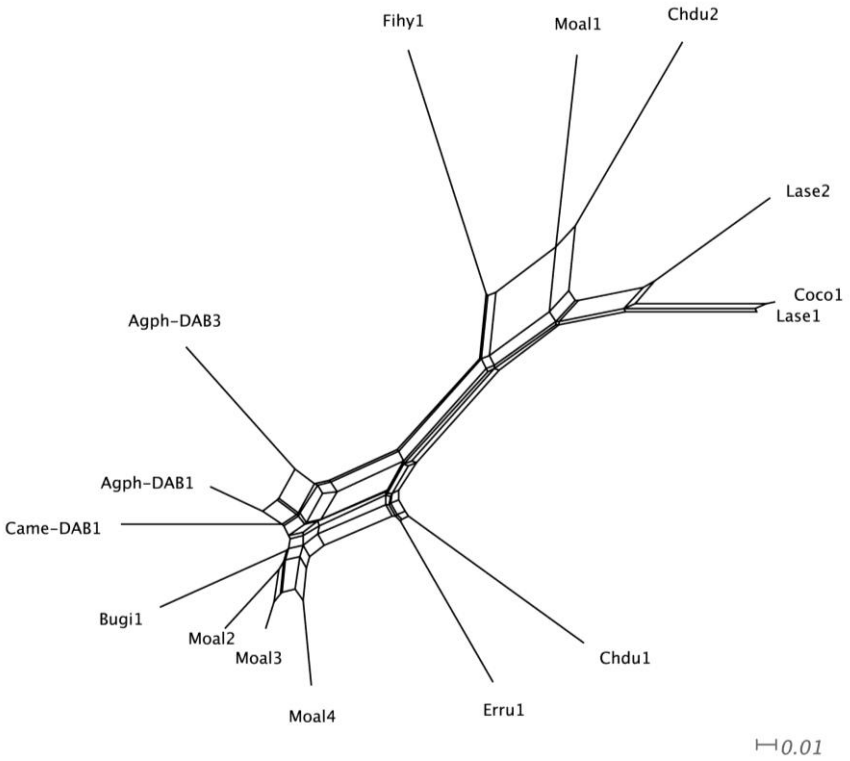


DISCUSSION

In this study we have for the first time isolated both coding and non-coding sequences corresponding to classical and non-classical MHC class II B genes in the pied flycatcher. The molecular protocol here described is also among the first ones demonstrating the utility of flanking intron sequences to simplify MHC genotyping in passerines. We show that intron sequences flanking the usually polymorphic exon 2 may assist the specific investigation of classical MHC class II B genes in species that, as passerines, are characterized by extensive gene duplication and pseudogenization (Westerdahl 2007). Importantly, classical and highly polymorphic MHC genes are the primary targets of pathogen-mediated selection (reviewed by Sommer 2005; Piertney and Oliver 2006) and the evasion of non-classical MHC genes with a more specific function and non-functional pseudogenes may accelerate data collection and diminish costs.

Our genetic data suggest the occurrence of at least 4 classical MHC class II B genes in pied flycatchers. However, suspicious evidence for chimera sequences makes this estimate far from conclusive. Additional studies more thoroughly minimizing PCR-mediated recombination (e.g. Lenz and Becker 2008, see also discussion below) and even genetic inheritance analyses should add more light in this respect. We expect our primers to be related with a very low or non-existent incidence of null alleles. Primers are located immediately in the introns-exon 2 junction and some constraints in the mutation of this important region involved in the splicing of mRNA are therefore expected. Moreover, previous studies in birds of prey have shown that exon 2-introns boundaries of homologous genes are well conserved within related species and even when comparing different raptor lineages (e.g. Alcaide et al. 2008, Burri et al. 2008a). The primers developed for the specific amplification of classical MHC genes in pied flycatchers also proved to cross-amplify MHC sequences in several passerine species of the Muscicapidae and Turdidae families (manuscript in prep.).

Figure 5. Neighbor-Net network of complete intron 1 sequences isolated in this study plus those isolated in the red-winged blackbird *Agelaius phoeniceus* (AF030997.1) and the house finch *Carpodacus mexicanus* (AF205032.1). The different sequences isolated within the same species are labelled with different numbers.



The cDNA sequences confirmed that both classical and non-classical MHC genes are expressed in pied flycatchers and are therefore functional. For classical MHC genes, we found an excess of non-synonymous substitutions, specifically for those amino acid positions that have been suggested to interact with antigens in the human MHC class II molecule (Brown 1993, see Figure 3). These regions have shown to accumulate positively selected sites in other avian lineages as well (e.g. Alcaide et al. 2007; Burri et al. 2008a, Silva and Edwards 2009). All these findings corroborate the

suitability of the classical MHC genes here described as relevant markers in eco-immunogenetics studies in the pied flycatcher.

Concerted evolution and the simplification of MHC-typing protocols in passerines

Even though we successfully evade the co-amplification of pseudogenes and non-classical genes, our specific primers for classical MHC class II genes co-amplify multiple loci. Both exon 2 coding sequences (Figure 3) and flanking intron sequences (data not shown) suggest that concerted evolution may be responsible for the homogenisation of the genomic sequence of classical MHC class II genes in pied flycatchers and other passerines (Westerdahl 2007). Concerted evolution is usually a considerable hindrance for the design of locus-specific primers (e.g. Alcaide et al. 2007) and this is the major reason behind our failure to design locus-specific primers in flycatchers. Under this scenario, sequencing the 3'-untransalted sequences (3' UTRs) of different genes has emerged as one of the very few alternatives to assign alleles to particular loci (Miller and Lambert 2004). Detailed characterization of the MHC class II B in the barn owl *Tyto alba* has nevertheless shown that certain genomic regions are gene conversion free (Burri et al 2008b), a fact that allowed researchers to design locus-specific primers. In the case of the jungle fowl (Worley et al. 2008), single locus typing at both MHC class I and class II loci was possible due to the comprehensive knowledge of the MHC of the conspecific domestic chicken *Gallus gallus*. Similar strategies can now also be applied to the recently characterized MHC of the zebra finch *Taeniopygia guttata* (Balakrishnan et al. 2010) and have already proven useful in red-winged blackbirds *Agelaius phoeniceus* (Gasper et al. 2001). Neither of these scenarios is presently feasible in the case of the pied flycatcher, although next-generation sequencing technologies are expected to revolutionize the characterization of MHC complexes in the near future (Genome 10K Community of Scientists, 2009).

An alternative to reduce the complexity of genotyping in those taxa with multiple classical genes may be the design of primers targeting only a subset of the allele repertoire, as explained in detail below.

Risk of chimera formation during the co-amplification of multiple loci

Our genetic data demonstrate that PCR-mediated recombination is a serious source of false or spurious alleles when a large number of alleles are co-amplified simultaneously. The implications of this phenomenon are critical since many of the most popular MHC-typing protocols, including next-generation sequencing approaches (Babik et al. 2009), rely on PCR amplification at some stage. Reducing the number of cycles and extending elongation times during PCR amplification have been suggested to diminish the confounding effects of *in vitro* recombination (Lenz and Becker 2008) and genotyping strategies in passerines may therefore consider these precautions thoroughly. Taking a look at our alignments (Figs. 2 and 4), the design of primers targeting only a subset of the allele repertoire could be an adequate alternative to reduce the co-amplification of large numbers of alleles. Variability in MHC originates to a large extent by recombining alleles exchanging particular nucleotide motifs. This is evident, for instance, across the 5' end of the coding sequence of exon 2 in pied flycatchers (Figure 3). Despite implying more tedious sample manipulation in the lab, these approaches (further supported by non-denaturing capillary electrophoresis (SSCP or RSCA; reviewed in Babik et al. 2009) may be a useful alternative to minimize the incidence of false and spurious alleles. Regarding denaturing capillary electrophoresis, recent research (Alcaide et al. 2010) has shown that the simultaneous analysis of multiple fragments enhances our capabilities to discriminate between alleles when compared to the analysis of single PCR amplicons. Thus, partial digestion of PCR amplicons with restriction enzymes could be a

promising strategy to improve resolution in cases similar to that documented here for the pied flycatcher. Alternatively, RSCA has also proven to be a very effective and high-throughput for the genotyping of duplicated MHC class II genes (Lenz et al. 2009).

Perspectives on additional passerine species

The cross-amplification of MHC sequences across a phylogenetically diverse array of passerine species decisively enhances the future applications of our molecular approach. Importantly, divergent introns-exon 2 boundaries within particular species such as the white wagtail, Dupont's lark or the woodchat shrine (Figure 2B) predict better opportunities for designing locus-specific primers or, at least, primers targeting a low number of loci. However, future studies in these and other species should tackle this issue in more depth to determine the broad utility of our protocol. We do believe that these data, although limited, may be really encouraging for the simplification of MHC-typing protocols in other passerine species since we have demonstrated that two single PCR reactions and the analysis of only a few clones are enough to isolate MHC sequences in the passerine species tested so far.

In some species, there was a trend for the preferential amplification of particular MHC fragments probably due to the large degeneracy of primers 326 and 325 (Table 1). For these reasons, we encourage the use of less degenerate primers (MHCIIPas-E2iF and MHCIIPas-E2iR) to minimize possible non-targeted products in passerines and biases towards the amplification of particular loci which may lead studies to miss important information on MHC structure. These primers were designed over conserved exon 2 motifs that emerged from an alignment of multiple passerine MHC class II sequences species. Nevertheless, these primers have not been tested in the present study and future studies will ascertain their utility. Finally, collection of genomic data will determine the suitability of similar approaches for

MHC class I genes in passerines. The vast majority of studies so far have nonetheless dealt with expressed genes and genomic data are therefore scant in this avian lineage (e.g. Westerdahl et al. 2004, Westerdahl 2007; Loiseau et al. 2009, Promerová et al. 2009).

Conclusions

This study highlights the advantages from the increasing knowledge in gene structure, polymorphism and expression profiles to simplify MHC typing protocols in passerine species. Importantly, the search for locus-specific primers opens the possibility to decisively overcome chimera formation and focus on computational inferences of gametic phase, one of the most promising alternatives for MHC genotyping in the future (Babik et al. 2009).

SUPLEMENTARY FILES

Suplementary file 1. Intron I sequences obtained from different passerines, other than the pied flycatcher, in reaction 1 (see Figure 1)

>**Agph-DAB1**----------
-GAGGGGCTGAGG-GGTGGGGGGAGGGGAGGGAGGAGTGAGA----------GAGGGGGAAAAGGAG--
AGGGGGGGACCCCAAAACTGAGCGG-GAGGGGGTCGGGGATTG-GGGAATT----------
TGTGGGAAAATGGGGAAA---------TGGGACCCCAAAAGTGATTT----GGGAGC--G----GTCGGAGGT---GGTAG-
GGGAGAGC---ATGTGGAAGGGGAAGTG-GGCGAA-
GGGCGGCAAAGAGGAAGCGGCAGCGCGGGCAGGAGGGTCCCGTGTCGGCC-----
GTGGGGCACAGGGGGTGCGGAGTGGGG-ATCCGGGGTGGGCCCCGGAGCTCTGGGGGTGC-------
TGCTGGGGGGTGCTG--GGGGGGCACCAC-
TGAGCTGTGTCCTGCACTCACAGGGGTGTTCCAGGAGATGCATAAGGTCGAGTGTTACTTCATTAACGGCACGGA
GAAAGTCAGGTTCGTGCAGAAGCTCATCTACAACCGGCTGCAGTACGCGATGTTCGACAGTGACGTGGGGCACTT
TGTGGGGTTCACCCCCTCTGGGGACATGAATGCCAAGCGCTGGAACAGCGACCCGGCCTTACTGGAGTACAAACG
GACTGCGGTGGACCGG----------

>**Lase1**----------
--------GGGATTCCCCCCGGCTACGGATGCG----------GAGCTGTCAGGTGAGC--AGGAGGGGT---AAGA---
AGTGGTGAGATCGTAG-GCACGGTGGG-AGA----------GAAGGGAAAAGGTGGAGG--------
TGGGACCACCAAACAGATGT-----GGGGGG------TCTGGCGAC---TGGGGACTTATGG---
GAAAGGGGGAAGAAAATATGGAGAA-
AGGTGGAAAAGAGGAAGTGGGGCCGTGGGCAGGAGGGTCTCATGGTGGCC-----
ATGGGACACAGGGGGTGCGGAGTGGGG-AGCCGGGGTGGCATCCGGAGCTCTGGGCTTGC-------

TGCTGGGGGGTGCTG--
GGGGGGCACCCCATGACCTGTGTCCCGCACAAACAGGGGTGTTCCGGGAGATGGGGAAGGTCGAGTGTCACTGC
ATTAACGGCACCGCCCGGGTGAGGTTGGTGCCGAGGCACATCCAACACCGGCAGCAGTGCGTGCACTTGGACAGC
GATGTGGGGCACTGTGGGGG---
CAGCCCGGATCGGGAGAAGGTTGCCAGGTGCCGGAACAGCCACCCGGAAAGGATGGAGAACATGTGGGCTGCT--
----

>**Lase2**----------
---------GAGAGTCCCCCCGGACACGGATGCA----------GAGCTGTCGGGTGAAC--AGGAGGGGC---ACGA---
AGTAGTGGGATGGTGGTGAATGGTGGGGAGA-----------GAAGGGAAAAGGTGGAAG--------
TGGGAACCCCAGACAGATG-----GGGGGGGGG----GCTGGCAAC---TGGAGAGTTATGG---
GAAAGGGGGAGGAAAATATGGAGGA-AGGTGGAAAAGAGAAAGTGGG-
CCACGGGCAGGAGGGTCTTATGGTGGCC-----GTGAGGCACAGGGAATGCGGAGTGGGG-
ATCCAAATTGGCCTCCGAAGCTCTGTGCTTGCTGGGGAGTGCTGGGGGCTGCTG--GGGGG-
CACCACTTGACCTGTCTCCTGCATACACAGGGGTGTTTCAGGAGATGGTTAAGACTGAGTGTCACTTCGTTAACGG
CACGGAGAAGGTGAGGTTGGTGGAGAGGCGCTTCTACAACCGGCTGCTACTCCTCATGTTCGACAGCGATGTGG
GGCACTACGTGGGGTTCACCCCGTATGGGGAGCTGATTGCCAGGAACTGGAACAGCGACCCGGTGATCATGGAG
CAAAGACGGGCCGCA----------

>**Moal1**----------
----------GAGCCCCCCCGGCTGCGGGCGCG----------GAGCTCTCGGGTGAGC--GGGGGAGGGGGAAGGGA--
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TGGGACCCTCGAATTGATGT---GGGGGGTG----GTGCTGGCGAC---CGAGGAGTTAT---
GGAAAAGGGGGAGGAAAATACGGAGAA-
GAATGGAAAAGACGAAGAGGGGCCGCGGGAAGAAAGGGCCTACTAGGGGTGGCACGTGGGGCATACGGGTTGC
ACAGTGTGG-ATCCGGGGTTGCC-TCTGAGATCTTGGTTTGCTGTGGGGTGCCGGCGGCTGCTG--
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TAACGGCACCGAGCGGGTGAGGTTCGTGAAGAGATTCATCTACAACCGGGAGCAGTACGTGCACTTCGACAGCGA
TGTGGGGCACTTTGTGGGGGACACCCCATATGGGGAGGAGGTTGCCAGGCACTGGAACAGCGACCCCGAATGGA
TGGAGCACAG----------

>**Moal2**-
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ATGGGGAAAAGGGGGCGAAGGGGGGAGCCCGGAATGGAGGGGGAGGAGATGGGGACCCCCCAAAGGGAGAG
CGGGAGGGGCTGAGG-GGTGGGGGTAGGGGAGGGAGGAGT-AGAG---------AGGGGGGAAAAGGAG-
AGGGGGGGACCCCCAAAACTGTGCGGGGAGGGGGTCGGGGGATTGGGGAATT----------
TTGGGGAAAATGCGGAAA---------TGGGACCCCAGAAGTGATTT----GGGAGCG------GCAGGAGGT---TGTAG-
GGGAGAGC---ATGTGGAAGGGGAAGTG-GGCGAA-
GGGCGGCAAAGAGGAAGCGGCAGCGCGGGCAGGAGGGTCCCGTGGCGGC------
GTGGGGCACAGGGGGTGCGGGGTGGGG-ATCCGGGGTGGGCCCCGGAGCTCTGGGGGTGC-------
TGCTGGGGGGTGCTG--AGGGGGCACCCC-
TGAGCTGTGTCCTGCACTCACAGGGGTGTTCCAGGAGATGCATAAGGTCGAGTGTTACTTCATTAACGGCACGGA
GAAGGTCAGGTTCGTGCAGAGGCTCATCTACAACCGGCTGCAGTACGCGATGTTCGACAGTGACGTGGGGCACAT
TGTGGGGTTCACCCCCTCTGGGGACATGAATGCCAAGCGCTGGAACAGCGACCCGGCCTTACTGGAGTACAAACG
GACTGC----------

>**Moal3-**GAGCCCCCCCCGGCTGCGGGCGCGGAGCTCTCGGGTGAGCGGGGGGCGGGAGGCGCTGGGAC-
GGGGGGGGAGAAGGGGGAAAAGGGGGGCGAAGGGGGGGAGCCCGGAATGGAGGGGAAGGAGGAGGGGACCCCCC
AAAGGGAGAGCGGGAGGGGCTGAGG-
GGTGGGGGGGAGGGGAGGGAGGGGTGGGAGAGGGTGGGGAAGGGGGAAAAGGAG-
AGGGGGGGGACCCCAAAACTGAGCAG-GAAGGGGCCGGGGATTG-GGGAATT----------
TTTGGGAAAATGGGGAAA---------AGGGACCCCAAAAGTGATTTG---GGGAGCG------GCCGGAGGT---AGGAG-
GGGAGAGG---GTGTGGAAGGGGAAATG--
GGGAAAGGGCGGCAAAGAGGAAGCGGCAGCGCGGGCAGGAGGATCCCGTGGCGGCC-----

GTGGGGCACAGGGGGTGCAGAGTGGGG-ATCCGGGGTGGGCCCCGGAGCTCTGGGG-TGC-------
GGCTGGGGGGTGCTG--GGGGGGCACCCC-
TGAGCTGTGTCCTGCACTCACAGGGGTGTTCCAGCTCCTGGCAAAGGCCGAGTGTCACTTCATTAACGGCACGGA
GAAGGTGAGGTTCGTGCACAGGCACATCTACAACCGGCTGCAGTACGCGATGTTCGACAGCGACGTGGGGCACTT
ACTGGGGTTCACCCCCTTTGGGGAGAGGGTTGCCAAGAACTGGAACAGCGACCCGGCCTTAATGGAGTACAAAC--
----

>**Moal4-**GAGCCCCCCCGGCTGCGGGCGCGGAGCTCTCGGGTGAGCGGGGGGCGGGAGGCGCTGGGAC-
GGGGGGGAGAAGGGGGAAAAGGGGGCGAAGGGAGGAGCCCGGAATGGAGGGGGAGGAGGAGGGGACCCCCC
AAAGCCANCGGAGGGTAGGGGTGGG-GGTAAGAGGAGGGGTGGGAGAGGTGGGA----------
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TTTGGGAAAATGGGGAAA---------TGGGACCCCAAAAGTGATTTG---GGGAGCG------GCCGGAGGT---AGGAG-
GGGAGAGG---GTGTGAAAGGGGAAATG--
GGGAAAGGGCGGCAAAGAGGAAGCGGCAGCGCGGGCAGGAGGGTCCCATGGCGGCC-----
GTGGGGCACAGGGGGTGCAGAGTGGGGGATCCGGGGTGGCCCCCGGAGCTCTGGGGGTCC-------
TGCTTGGGGGTGCTG—
GGGTGGCACCACCGGACCTGTGTCATGGACATTCAGGGATGTTTCAGGCGGTGGCAAAGCAGGAGTGTTACTTCA
TTAACGGCACAGAGAAGGTGAGGTATGTGCTGAGGTACATCTACTACAGGGAGCCATACGCGACGTTCGACAGCG
ACGTGGGGCACTACGTGGGGTTCACCCCCTATGGGGAGAGGAATGCCAAGCGCTTGAACAGCGACCCCGCCTTAC
TGGAGGACAGACGGGCTTCGGTGGACAGATACTGCCGACAC

>**Chdu1**----------
GAGCGGCGCCGGCTGCGGGCGCGGAGCTGTCGGGTGAGCGGGGCCGG-GGCGGGAGGCGCTGGGCCAGGGG-
GGGAG---------AAGGGGGAAAAGGGG---AGGGGGGACCCCAAAAGTGAGCGG-
GAGGGGGCTGGGGATGGGGGGGGATTGAGGTAAAAATGTTTGGAAATGGGTAAAAGTGGGGATTGGGAGCCCG
GAAGGGATTT----GGG-GCGTG----TCCGGAGGT---GGGAG-GGGAGGGG---
CCGGGCAAGGGGCAATGGTTTGGGCGGGCGGAGAAGAGGAAGCGTTGGCGCGGGCAGGAGGGGCC-

ATGGCGGCC-----GTGGGGCACAGGGGGTGCGGAGCGGGG-ATCCGGGG-GGGCCCCGGAGCTCTGGGGGTGC----
-------TGGGGGTG------
GGGGGCAGCCCCTGAGCGGGGTCCTGCACACTCAGGGTGGTTCCAGCGCATGATGAAGGCCGAGTGTCACTTCA
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GACGTGGGGCACTACGTGGGGGACACTCCCTTTGGGGAGATCCAGGCCCGGTACAAGAACAGCGACCCGGATTT
CATGGAGCAGAGACGGGCTCA----------

>**Chdu2**----------
----------GAGCCTCCTTGGTTGCGGACGCG----------GAGCTCTCAGGTGATC--AGGAGGGGCGGAAGGAA--
AGTGGTGGGAGGGTGG-GAACGGTGGGGCAT-----------GGAGGGAAAAGGGGGAAG--------
TAGGGCCCTCAAATTGATGTTTTGGGGGGCGGGTGGTGCTGGCGACTGAGGAGGGGTGCT-G-
GCGAAAGAGGGAGGAAAGTATGGAGAA-
GGGTGGAAAACAGGAAATGGGACCGCGGGAAGAAGGGTCCTACAAGGGTTAGCATGTAGGGCGCCAGAGGTGC
AGAATGGGG-ATTCGGAGTGGCCCTCTGAGCTCTTGGCTTGCTGTGGGGTGCTGGGAGCTGCTG--
GTGGGGCACCTCTCGATCTGTCTTATGCATGCACAGGGGCGTTCCAGGAGATGGTTACGTCCGAGTGTTACTTCAT
TAACGGCACCGACCGGGTGAGGTTTGTGAAGAGGTTCATCTACAACCGGGAGCAGTACGTGCACTTTGACAGCGA
CATGGGGCTATATGTGGGGGACACCCCATATGGGGAGAAGGTTGCCAGGTACTGGAACAGCGACCCGGAATGGA
TAGAGTATAGACGGGATGC----------

>**Bugi1**GGAGCCCCCCCGGGGGCGGGCGCGGAGCTCTCGGGTGAGCGGGGGGCGGGAGGCGGTGGGACAGGGG
GGGAGAAGGGGGAAAAGGGGGCGAAGGGGGGAGCCCGGGATGGAGGGGGAGGAGTTGGGGACCCCCCAAAG
GGAGAGCGGGAGGGGAGGGAGGGGTGGGAGAGGGTGGGGAAGGGGGGAAA----------AGGGGAGGGGAG----
-----GACCCCGAAACTGAGCCA-GAGGGGGGTGGGGATTGGGGGAATT----------
GGGACCCCCAAAGTGTTTCG---GGGATCA------GCCGGAAGT---GGGAG-GGGAGAGG---
ATGGGGAAGGGGAAATGGC--
GGAAGGGCGGCGCAGAGGAAGCGGCAGCGCGGGCAGGAGGGTCCCGTGGCGGCC-----
GTGGGGCACAGGGGGTGCGGAGTGGGG-ATCCGGGCTGGGGCCCGGAGCTCTGGGGGTGCTGCTGGG----

GGGGGGTGCTGGGGAGGGGCACCCCCTGAGCTCTGTGCTGCTCTCACAGGGGTGTTCCAGGCGATGGTAAAGG
GCGAGTGCCACTTCACTAACGGCACGGAGAAGGTGAGGCTCGTGGTGAGGTACATCTACAACCGGCAGCAGCTC
GTGGTGTTCGACAGCGACGTGGGGCACTACGTGGGCTTCACCCGCTTTGGGGAGATGAATGCCGAGCGCTGGAA
CAACAACCCGGCCATAATGGAGAATGAACGGACTGC----------

>**Erru1**----------
GAGCCCCCCCTGGGGCGGGCGCGGAGCT-CTGGGTGAGCGGGG--GG-GGCGGGAGGCGGAGGGACAGGGG--
GGAG---------ATGGAGGAAAATTGG---GGGTTAGACCCCAAAATTGAGCGGGAAGGAATCTCGGTATTCCGGG--------
--AAAACGATGGGAAAGGGG-AAAAGGGGGAAATGGGACCCTAAAAGTGATTTT---GGG-ATTTG-----CTGGAGGA---
TGGAG-GGGAGAGG---ATGTGAAAGCGGAAATGGG--
GGAAGGGTGGAGAAGAGGAAGAGGCAGCGCGGGCAGGAGGGTCCCATGGCGGCC-----
GTGGGGCACAGGGGGTGCGGGGTGGGG-ATCCGGGG-TGGCCCCGGAGCTCTGGGGGTGC----------TG-----
GGGGGGCTCCAACTGAGCCGTGTCCGGCACACACAGAGGTGCTGCAGGAGTTTCGCACAGCCGAGTGTCACTTCA
TTAACGGCACGGAGAAGGTGAGGTTGGTGGAGAGACGCTTCTACAACCGGTTCGAGTACGCGAGGTTCGACAGC
GACGTGGGGCGGTTCGAGGGGTTCGACCCCTATGGGGAGAGACAGGCCCAGTACTGGAACAGCAACCCGGATAT
AATGGAGAACAGACGAACAGCT----------

>**Coco1**----------
----------GATTCCCCCCGGCTACAGATGCG----------GAGCTGTCAGGTGAGC--AGGAGGGGT---AAGA---
AGTGGTGAGATCGTAGGCAACGGTAGGGAGA----------GAAGGGAAAAGGTGGAGG--------
TGGGACCACCAAACAGATGT-----GGGGGG------TCTGGCGAC---TGGGGACTTATGG---
GAAAGGGGGAAGAAAATATGGAGAA-
AGGTGGAAAAGAGGAAGTGGGGCCGTGGGCAGGAGGGTCTCATGGTGGCC-----
ATGGGACACAGGGAGTGCGGAGTGGGG-AGCCGGGGTGGCATCCGGAGCTCTGGGCTTGC----------
TGGGGGCTGCTG--
CGGGGGCACCCCATGACCTGTGTCCCGCACAAACAGGGGTGTTCCAAGAGATGGTTAAGTCTGAGTGTCACTTCA
TTAACGGCACCGCCCGGGTGAGGTTTGTGAAAAGGTTCATCTACAACCGGGAGCAGTATGTGATGTTTGACAGCG

ATGTGGGGGTGTTTGTGGGGGACACCCCGTATGGGGAGAAGGTTGCCAGGTACTGGAACAGCGACCCGGAATG
GATGGAGTACAGACGGGCTGC----------

>**Phco1**----------

GGGGGGGCAACCCCTGACCTGTGACCTGCACAGCCAGGGGTGTTCCAGGAGATGGTAAAGCGCGAGTGTCACTT
CATTAACGGCACGGAGAAGGTGAGGTACGTGGACAGGTACATCTACAACCGGGAGCAGTTCGTGATGTTCGACA
GCGACGTGGGGCTGTTTGTGGGGGACACTCCCGCTGGGGAGAAGTGTGCCAGCAACTGGAACAGGCAACCGGAA
ATACTGGAGTTCAAACGGGGTGAA----------

>**Syme1-**----------

CAGGGGTGTTCCAGGAGATAGTTAAGTCCGAGTGTCACTTCATTAACGGCACCAACCAGGTGAAGTTTGTGAAGA
GGTTCATCTACAACCGAGAGCAGTATGTGCACTTCGACAGCGATGTGGGGCTGTACGCGGGGGACACCTCATATG
GGGAGAAGGTTGCTAGGTACTGGAATAGCGACCCAGAATGGATGGAGTACAGACGGGATGCA----------

Suplementary file 2. Intron II sequences obtained from different passerines, other than the pied flycatcher, in reaction 2 (see Figure1)

>**Coco1**----------
TATCTTGCCCCCCCCCAGTGACCCCCATCC-CTCTCTTTGTCCATTCCAGT---
CCATCCCAGTCTCTCCCAGTCACTTCCACTCCATTCCCAGTCCAGTCCATCCCAGTCTCTCCCAGTCACTTCCACTCCA
TTCCCAGTCCCTCTCAATGCCA-
CACGAAACCTCACCGCTCTCTCCCAGTGCCCCCCAGCGTGTCCATCTCGCTGGTGCCC---
TCGAGCTCCCAGCCTGGCCCTGGCCGCCTGCTGTGCTCCGTGATGGATTTCTACCCTGCGCAGGTGCAGCTCAGGT
GGTTCCAGGGCTGGCCGGAGCTCTTGGGGCACGTGGTGGCCACCGACATTGTCCCCAACGGGGTCTGGACCCACC
AGCTCCTGGTGCTGCTGGA

>**Moal1**----------TCCACTTTCAG--
ACTTCCATCCTCTCTCCCAGTGCCCTCC----------CAGCGTTCTCCAACCCCCTCC----
CAGTCTCTCCCCAGTCCCTCTCAATGTTACCTGAGTCCTTCCCCGTGTCTCCCATTTCACCCGCATTTTATCTCGGAG
TGTCCCTGCTCTCTCCCAGTGCCCCCCAGCGTGTCCATCTCGCTGGTGCCC---
TCGAGCTCCCAGCCCGGCCCCGGCCGCCTGCTCTGCTCCGTGATGGATTTCTACCCTGCTGCCATCCAGGTGAGGT
GGTTCCAGGGCCAGCAGGAGCTCTC------
TGTGTTGGCCACCGATGTGGTCCCCAACGGGGACTGGACCTACCAGCTCCTGGTGCTGCTGGA

>**Syme2**----------
ATGCGGGGCTCTCGGCCCCCCAGTGACACGCGCTTCGTTCCCAGGTCCTCTCACTTCATTCCCATTCCATTCCCAGT
CTTTCTCAGTCCGCTCCCAG--ACCTTCATCCTCTCTCCCAGTGGTCTCCAGT---

TCAGTCCCATCTCTTTCAGTACCCTCC----CAGTTCCCCCCCGGTCCTTCTCAGTGTTACCCGAGCCCTCCCCCGA--
CTCCCATTTTATCCGTATCTCATCCCAGTGTCTCCTTGCTCTCTCCCAGTGCCCCCCAGCGTTTCCATCTCGCTGGTG
CCC---
TCGAGCTCTCGGCCCGGCACCAGCCGCCTGCTGTGCTCTGTGATGGATTTCTACCCCGCCCACATCCAGGTGAGGT
GGTTCCAGGGCCAGCAGGAGCTCTCGGGACACGTGGTGGCCACCGACGTGGTCCCCAACGGGGACTGGAGCTAC
CAGCTGC----------

>**Bugi1**----------

GTGTCCATCTCCCTGGTGCCCCCCTCGGGCTCCCAGCCCGGCCCCGGCCGCCTGCTCTGCTCCGTGATGGATTTCTA
CCCTGCCCCAATCCAGGG-
AGGTGGTTCCAGGGCCAGCAGGAGCTCTCGGAGCACGTGGTGGCCACCGACGTGGTCCCCAACGGGGACTGGAC
CTACCAGCTGCTGGTGCTGCTGGA

>Lase1CCCGGAATGGACGGAGAACATACGGGCTGCTGTGGACAGGTGCTGCTGGTCCAGCTACGAGTTGTCCACC
CCCTTCCTCGTGGGCCGCAGAGGGGAGCGTGGGGCAGAGCGTGTCCCCTCGGGCCCTGCCTTGGGAATGACCCT
GGAGCCCCTCCAAACCTCCCTGGGAATCACCCCAGAGCCCTCAGCCCTCCTTGAGCCCATCCCCAGGGCCTCTTGTC
CCA---CCGCCCTGTGACTCCCGTGCCTCTCCCAGTCTGTCCCAGTCCC----------TCTGTG-TCCCTTCCAGTGCCCCC-
AGTGTGCCCAATTCTCTGGTGCCC---
TCGAGCTCCCTGCCCGGCCCCGGCCGCCTGCTCTGCTCCATGATGGACTTCTACCCTGTGCGGGTGCAGCTCAGGT
AGTTCCAGGGCCAGCAGGAGCTCTCGGGGCACGTGGTGGCCACCGACATTGTCCCCAACGGGGACTGGAGCCAC
CAGCTCCTGGTGCTGCTGGA

>**Chdu1**----------

CCTGCTGTGCTCCGTGATGGATTTCTACCCTGCGCACATCCAGGTGAGGTGGTTCGAGGGCCAGCAGGAGCTGTC
GGAGCACGTGGTGAGCACGGACGTGGTGGCCAACGGCGACTGGAGCTGGCAGCTGCTGGTGCTGCTGGAAAGG
CCGCCCCGGCGCGGGCTCAGCTACACGTGCCAG

SÍNTESIS. FUTURAS INVESTIGACIONES

El papamoscas cerrojillo es una de las especies de aves mejor estudiadas a lo largo de Europa. Sin embargo, son muchas las cuestiones aún sin resolver en relación al sistema reproductivo de la especie o al impacto que la variabilidad genética tiene en la aptitud individual. La presente tesis avanza en el entendimiento de los mecanismos que promueven la poligamia (principalmente debida a paternidad extra pareja, EPP) y en las consecuencias que ello tiene en el éxito reproductivo de los individuos, así como en los caracteres sexualmente favorecidos. Además, mediante la combinación de técnicas moleculares y de captura-recaptura, se ha evaluado el papel de la variabilidad genética neutral en la supervivencia a lo largo de la vida de los individuos. El estudio se ha llevado a cabo en una población ibérica que presenta diferencias, tanto a nivel fenotípico como genético, con el resto de poblaciones centro y norteuropeas (Haavie y col. 2000; Lehtonen y col. 2009). Asimismo, durante la tesis, se han desarrollado nuevos marcadores neutrales (microsatélites, **Capítulo I**) y sometidos a selección (MHC, **Capítulo VI**), que suponen una valiosa aportación para abordar futuras cuestiones ecológicas y/o evolutivas.

El desarrollo de marcadores y el abaratamiento de las técnicas moleculares han permitido que los análisis genéticos sean hoy comunes. Sin embargo, el potencial de estas herramientas para responder cuestiones evolutivas aumenta notablemente cuando va acompañado de información ecológica apropiada. En este sentido, la información recogida durante las campañas de campo ha resultado esencial para contestar a las cuestiones planteadas en la presente tesis. El seguimiento de la población (desde 1984) ha permitido estimar parámetros como la calidad de los territorios de cría (basándonos en la productividad y tasa de ocupación de éstos durante los últimos 15 años; **Capítulo III**) o la supervivencia y longevidad de los individuos procedentes de las cohortes de 2005 y 2006 (**Capítulo V**).

Hablando de poligamia

Hoy se sabe que, en aves socialmente monógamas, cierta frecuencia de paternidad extra pareja es la regla más que la excepción (Griffith y col. 2002; Westneat y Stewart 2003). Este fenómeno tiene importantes implicaciones evolutivas; entre las estudiadas aquí: incrementa 1) la varianza en el éxito reproductivo; 2) las presiones selectivas sobre los caracteres favorecidos sexualmente; y 3) la intensidad de los mecanismos relacionados con la pérdida o ganancia de paternidad. Uno de esos mecanismos es la protandria, esto es, la llegada temprana de los machos respecto a las hembras a los territorios de cría tras la migración primaveral (Kokko y col. 2006). Una fenología de cría temprana repercute en un mayor éxito reproductivo (Newton 2008). Sin embargo, llegar pronto puede acarrear costes debido, por ejemplo, a condiciones ambientales adversas durante la migración o en los territorios de cría (Brown y Brown 2000). Así, los individuos se enfrentan a un compromiso entre los beneficios y costes asociados a llegar pronto, de forma que la protandria debería aparecer cuando los primeros son mayores a los segundos (Kokko 1999). En aves, se piensa que esos beneficios se deben, principalmente, a la mayor probabilidad de ser polígamo (mediante EPCs y/o poligamia social, *"mate opportunity" hypothesis;* Wiklund y Fagerström 1977) y/o a la obtención de un territorio de alta calidad ("*rank advantage" hypothesis*; Keterson y Nolan 1976), circunstancias que repercuten en el éxito reproductor individual. Trabajos previos han demostrado la importancia de una u otra hipótesis en especies territoriales (ej. Myers 1981) o polígamas (Lozano 1996; Reuding y col. 2009). Sin embargo, el **Capítulo II** es el primero en considerar simultáneamente todos los factores implicados en ambas hipótesis (EPP, poligamia social y calidad del territorio). Los resultados muestran que la probabilidad de ser polígamo (social y o genético) disminuyó con el avance de la estación, es decir, los machos que criaron temprano tuvieron más probabilidades de ser polígamos, hecho que repercutió positivamente en su éxito reproductivo. En contra, la calidad del territorio (estimada en base a su

productividad y tasa de ocupación durante los últimos 15 años) no estuvo relacionada con la fecha de cría como cabria esperar si el territorio obtenido dependiese del número competidores que llegaron antes. Por otro lado, sería lógico pensar que los mejores territorios son ocupados por individuos de alta calidad (Alatalo 1986). Sin embargo, esta relación tampoco parece darse en la población de estudio ya que los machos de mayor tamaño o con manchas frontales mayores (rasgos favorecidos en la competencia intrasexual por territorios; Lundberg y Alatalo 1992; Sanz 2001) no ocuparon los mejores territorios. A diferencia de los machos, en las hembras no parece existir una presión selectiva tan fuerte por llegar pronto ya que las probabilidades de tener EPP no variaron con la fecha de puesta, aunque sí aumentaron las de ser secundarias. Si bien ser secundaria acarrea costes en el éxito reproductivo anual (ej. Huk y Winkel 2006), algunos estudios sugieren que esto podría ser contrarrestado por un mayor éxito reproductivo de la descendencia (Ligon 1999; explicado más adelante).

Pero, ¿por qué una llegada temprana tras la migración aumenta la probabilidad de ser polígamo? Dos factores son clave para entenderlo: i) El escaso periodo fértil de las hembras en aves. En concreto, en el papamoscas cerrojillo (y collarino (*F.albicollis*), su especie hermana) se estima que la fase fértil va desde 2 días antes de poner el primer huevo hasta que el penúltimo huevo es puesto (con un pico de fertilidad entre los días -2 y +1 respecto a la fecha de puesta del primer huevo; Lifjeld y col. 1997; Michl y col. 2002). ii) El compromiso en paternidad al que se enfrentan los machos debido a los beneficios de ganar paternidad fuera del nido frente a los costes que supone estar alejado del nido (perder paternidad). Una estrategia ideal para los machos sería, por tanto, proteger a la hembra social durante su pico de fertilidad y buscar EPCs después, lo que permitiría maximizar los beneficios (ganar paternidad) frente a los costes (perderla; Birkhead y Biggins1987). Los resultados encontrados en el **Capítulo IV** sugieren que los machos intentan seguir tal estrategia puesto que, pese a tener hembras accesibles alrededor, la mayoría de los machos incurrieron en EPP una

vez que su hembra social dejó de estar fértil. El problema es que el número de hembras fértiles disminuye con el avance de la estación, y que ese descenso es aún mayor en años con una alta sincronía de cría donde un gran número de hembras dejan de estar fértiles en unos pocos días. Así, los machos que lleguen simultáneamente o más tarde que las hembras perderán oportunidades de ganar EPP de forma proporcional al número de hembras que dejan de estar fértiles en la población cada día (Kokko y col. 2006). La consecuencia es que, únicamente los machos que crían temprano logran maximizar el compromiso en paternidad (**Capítulos III y IV**) ya que, por un lado, tienen pocos competidores durante el pico de fertilidad de su hembra social (pocas probabilidades de perder paternidad) mientras que, por otro, cuando su hembra deja de estar fértil, todavía hay un gran número de hembras por llegar y/o fértiles alrededor (muchas probabilidades de ganar EPP). Este resultado ayuda además, a explicar por qué la mayoría de los individuos que ganaron EPP no la perdieron en su nido (**Capítulo II**). Una vez que los individuos estuvieron en el lugar y momento adecuados, el fenotipo del macho determinó el éxito en EPP. Como demostramos en el **Capítulo II**, aquellos machos que ganaron paternidad fueron de mayor tamaño y más atractivos (con plumajes dorsales más negros y manchas blancas frontales mayores) que los machos de las hembras que incurrieron en relaciones extra pareja. En resumen, nuestros resultados sugieren por tanto, que el éxito en EPP es consecuencia de sucesos encadenados: llegar pronto, criar pronto y ser atractivo permite a esos individuos ganar paternidad y, mayoritariamente, evitar perderla. Por otro lado, dado que ganar paternidad aumentó el éxito reproductivo de los individuos (**Capítulo II y III**), la selección sexual debe estar potenciando la evolución de los rasgos sexualmente seleccionados.

Un interrogante es si, en última instancia, el éxito en EPP es consecuencia de una mayor predisposición por parte de las hembras a tener EPCs con machos de mayor tamaño y ornamentos más atractivos o, por contra, se debe a una mayor habilidad por parte de esos machos para forzar o conseguir EPCs. Probablemente, el

resultado final no responde ni a una ni a otra razón, sino que es consecuencia de la interacción entre el macho extra pareja, la hembra, su macho social y los factores ecológicos que los rodean (**Capítulo III**). De hecho, un reflejo de esas interacciones que evidencia el conflicto de ambos sexos ante la paternidad es que: 1) los machos no incurren en EPP con la hembra fértil más cercana; 2) No todos los machos que ganan paternidad evitan perderla. Esos resultados sugieren que algunos machos pueden ser muy eficaces custodiando a las hembras, y/o que machos de alta calidad pueden ser vecinos de otros de calidad aún mayor (frente a los que podrían perder paternidad), un hecho que parece corroborar la ausencia de diferencias fenotípicas a nivel poblacional entre los machos que ganan paternidad y los que no la ganan (es decir, los machos extra pareja no son más atractivos que la media poblacional). Estudios experimentales o el uso de técnicas como el *radiotracking* son necesario para esclarecer si el éxito en EPP viene determinado en última instancia por la conducta del macho o si en cambio, existe una elección femenina. Aunque, sin duda, el test más trascendente para testar si las EPC son adaptativas para las hembras (es decir, si obtienen beneficios a nivel genético) es la comparación entre medio-hermanos maternos dentro de la misma nidada. Si hay elección femenina, debe haber una variación en el beneficio neto obtenido tras ello (que haya permitido evolucionar a este mecanismo, es decir, ser adaptativo). En referencia a los medio-hermanos maternos, si ambos tipos de pollos crecen en el mismo ambiente, las diferencias en eficacia biológica podrían atribuirse a la contribución genética por parte del padre extra pareja (aunque los efectos maternos podrían confundir esa conclusión; Griffith y col. 2002). Numerosos estudios han llevado a cabo este tipo de comparación con resultados ambiguos (revisado en Akçay y Roughgarden 2007). Un hecho a resaltar es que la mayoría han buscado diferencias en las etapas tempranas de la vida (durante el nido o hasta el reclutamiento) en rasgos relacionados con la aptitud (inmunocompentencia, Johnsen y col. 2000 o tamaño corporal, Bouwman y col.2007; ver apéndice Akçay y Roughgarden 2007). Sin embargo, el test definitivo para validar un posible beneficio genético derivado de

EPCs es comparar el éxito reproductivo de los pollos EPY con el de sus medio-hermanos a lo largo de toda la vida (*lifetime reproductive success*). Esto sólo se ha realizado, hasta la fecha, en unos pocos estudios (Schmoll y col. 2009; Brouwer y col. 2010; Sardell y col. 2010; Gerlach y col. 2011) siendo necesarios mucho más trabajos de este tipo para arrojar luz sobre lo adaptativo de este comportamiento para las hembras. Algo similar ocurre en la poligamia social, donde todavía no está claro si las hembras secundarias compensan el efecto negativo a corto plazo que supone emparejarse con un macho polígamo (Gustafsson y Qvarnström 2006). Para los machos, ser polígamo social parece ser poco costoso pues no se suelen emparejarse muy lejos del nido primario (Potti y Montalvo 1993), y la asistencia a hembras secundarias suele ser pequeña o inexistente (Alatalo y Lundberg 1984b, Potti y Montalvo 1993). Qué lleva a las hembras a emparejarse con machos ya emparejados es, en cambio, un interrogante. Parece que la llegada tardía los territorios de cría, típica de estas hembras (Lundberg y Alatalo 1992, **Capítulo III**), las obliga a emparejarse apresuradamente. Sin embargo, no está claro si los machos ocultan su estatus a las hembras (deception hypothesis; Alatalo y Lundberg 1984) o si éstas se emparejan con machos polígamos en busca de un beneficio genético. Hay evidencias que sugieren que los machos polígamos son de alta calidad (llegan los primeros de la migración prenupcial (**Capítulo III**) y exhiben el plumaje más vistoso; Gustaffson y col. 1995), por lo que las hembras podrían beneficiarse si, por ejemplo, la descendencia heredara el atractivo del padre (*sexy son hypothesis*; revisado en Ligon 1999). Los estudios que se realicen en el futuro deberían explorar el éxito reproductivo de la descendencia de hembras secundarias a lo largo de la vida para intentar esclarecer todas estas cuestiones.

Hablando de variabilidad genética

Cuando individuos relacionados genéticamente se emparejan, su descendencia suele disfrutar de una menor eficacia biológica debido a las mayores probabilidades de

expresar alelos recesivos deletéreos (*inbreeding depression*; Charlesworth y Charlesworth 1987). Entender el papel de la diversidad genética en la aptitud individual tiene, por tanto, una gran relevancia en biología evolutiva (ej. revisado en Keller y Waller; Kempeaners 2007). Esto, por ejemplo, nos puede ayudar a comprender la evolución de la elección de pareja (y mecanismos afines a ella: EPP, mecanismos de elección post-copula), es decir, a esclarecer qué lleva a elegir o descartar a unos individuos respecto a otros como pareja. Por extensión, ese conocimiento tiene, además, importantes aplicaciones en la conservación de especies amenazadas (Hedrick y Kalinowski 2000), donde conocer la carga endogámica de los individuos y sus consecuencias en la aptitud individual es esencial para un manejo exitoso de poblaciones amenazadas (ej. mediante el cruce de individuos que maximicen la diversidad alélica).

Una aproximación que se ha popularizado en los últimos años debido a las dificultades de reconstruir genealogías fiables en poblaciones naturales es estimar la diversidad genética en base a un puñado de marcadores neutrales y luego, correlacionarlo con aspectos afines a la eficacia biológica. En el **Capítulo VI,** usamos esta aproximación para evaluar la influencia de la diversidad genética en la supervivencia de los individuos. La mayoría de estudios han explorado la relación entre heterocigosidad y supervivencia en la fase embrionaria, durante la vida en el nido o hasta el reclutamiento de los individuos (revisado en Coltman y Slate 2003; Chapman et al. 2009) dada la dificultad de registrar la supervivencia de los individuos en poblaciones naturales. Si bien, es esperable que las máximas diferencias en la supervivencia de los individuos ocurran en etapas tempranas de la vida, es también esencial estudiar los posibles efectos de la diversidad genética en la etapa adulta, ya que, en caso contrario, éstos podrían ser subestimados (Grueber y col. 2010) o incluso no llegar a ser detectados (Hardenber y col. 2007). Nuestros resultados, en contra de lo esperado, sugieren que la heterocigosidad medida en base a 15 microsatélites no está relacionada con la supervivencia hasta el reclutamiento ni durante los años

subsiguientes. Entre los argumentos que podrían explicar esta falta de relación, podríamos considerar que los factores ambientales que los individuos encuentran tras salir del nido hayan contrarrestado cualquier efecto de la heterocigosidad en la aptitud o que las diferencias en supervivencia en relación a la heterocigosidad ocurran en fases previas a las medidas (ej. durante fase embrionaria). Sin embargo, por encima de todos ellos cabe destacar la posibilidad de que este tipo de aproximación, al ser de carácter indirecto, sea un pobre estimador de la diversidad genética adaptativa ya que por lo general, se busca que la heterocigosidad de los marcadores refleje el estado de genes funcionales a nivel del genoma o, al menos, que alguno de los loci esté ligado a otro bajo selección. Un avance importante será, por tanto, cuantificar directamente la diversidad de genes biológicamente significativos, es decir, aquélla sometida a selección, y ver cómo ésta afecta a la eficacia biológica de los individuos. En este sentido, hemos desarrollado, a partir de regiones conservadas (de rapaces) del MHC clase II, cebadores específicos (de regiones no codificantes) que permiten amplificar preferencialmente genes polimórficos, así como evitar la de pseudogenes (genes que han perdido la capacidad de expresarse) y genes monomórficos (**Capítulo VI**). Los genes del MHC son genes de gran interés biológico pues juegan un papel principal en la respuesta inmunitaria. Estos genes presentan péptidos foráneos a células T especializadas, encargadas de desencadenar la respuesta inmune (Klein 1986). Se piensa que el mantenimiento de la tremenda diversidad alélica del MHC, la más alta registrada entre los genes funcionales de los vertebrados (Robinson y col. 2003), se debe a diversos procesos que conducen a selección balanceante (Sommer et al 2005). Además, la selección sexual puede ser un factor adicional en el mantenimiento de la alta diversidad alélica ya que estos genes juegan un papel importante en la selección de pareja en algunas especies (Milinski, 2006). Sin embargo, aún estamos lejos de comprender qué procesos evolutivos y ecológicos generan y mantienen la diversidad del MHC en poblaciones naturales (Edwards y Hedrick, 1998) o cómo de crucial es la variación en el MHC para la respuesta inmune o para la eficacia biológica en general.

Esta falta de conocimiento es aún mayor en el orden de los paseriformes, donde el estudio de la variabilidad de MHC a nivel individual se limita unos cuantos trabajos (ej. Westerdahl y col. 2005, Bonneaud y col. 2006 a,b, Westerdahl 2007, Whittaker y col. 2012). Ello se debe, sin duda, a los problemas en el desarrollo de metodologías capaces de sortear las dificultades derivadas de la complejidad génica del MHC, consecuencia de procesos como la duplicación y conversión génicas o presencia de pseudogenes (Babik y col. 2009). El desarrollo de nuestro protocolo abre las puertas a futuras investigaciones sobre la variabilidad individual del MHC y la aptitud individual en el orden de los paseriformes.

CONCLUSIONES

1. La obtención de un territorio de alta calidad y/o la probabilidad de aparearse un mayor número de veces son los factores principales que promueven la llegada temprana a las áreas de cría de los machos respecto a las hembras tras la migración. En la población de estudio, la calidad del territorio no estuvo relacionada con la fecha de cría (usada como una aproximación de la fecha de llegada) ni tuvo influencia en contextos de poligamia (social o vía extra pareja). Por contra, en el caso de los machos, las posibilidades de convertirse en polígamos (social o vía extra pareja) decayeron con el avance de la estación, mientras que para las hembras, las probabilidades de incurrir en paternidad extra pareja no variaron a lo largo de la temporada de cría, esas de ser secundaria aumentaron. Aunque ser secundaria tiene un impacto negativo en el éxito reproductivo anual, éstas podrían mejorar su eficacia biológica en las generaciones futuras a través de un mayor éxito reproductivo de su prole (por ejemplo, si heredan el atractivo del macho polígamo).

2. El éxito reproductivo de los machos disminuyó con el avance de la temporada de cría en los dos años de estudio. Ésto, influido en parte por el número de pollos sacados en el nido social, se debió principalmente al impacto positivo que ser polígamo (social y/o genético) tuvo en el éxito reproductivo anual de los machos. Así, nuestros resultados (ver **conclusión 1**) sugieren que la llegada temprana a las áreas de cría de los machos respecto a las hembras tras la migración se encuentra favorecida por selección sexual.

3. Los machos se enfrentan a un compromiso consistente en ganar paternidad a través de EPCs en otros nidos y tratar de asegurar la suya en el nido social. En nuestra población, la mayoría de los eventos extra pareja ocurrieron durante la puesta e

incubación de la hembra social del macho extra pareja, a pesar del gran número de hembras fértiles que hubo antes y después de esos periodos. Parece existir, por tanto, una estrategia por parte de los machos para maximizar su éxito reproductor basada en custodiar a la pareja social durante su pico de fertilidad (días previos a la puesta) y buscar EPCs después de ello.

4. A raíz de las **conclusiones 2** y **3**, se desprende que los machos que crían pronto respecto a sus vecinos son capaces de resolver el compromiso en paternidad, ya que minimizan los riesgos de perderla (cuando su hembra esta fértil hay pocos competidores) a la vez que maximizan los de ganar EPP (cuando su hembra deja de ser fértil todavía quedan un gran número de hembras fértiles por llegar de la migración).

5. La mayoría de hembras establecieron paternidad extra pareja con machos ya emparejados (ver arriba), de mayor tamaño y con plumajes dorsales más oscuros y manchas frontales mayores que los de sus parejas sociales. El fenotipo del macho parece jugar, por lo tanto, un papel fundamental en la probabilidad de obtener paternidad extra pareja. Puesto que ganar paternidad extra pareja incrementó la varianza en el éxito reproductivo de los machos, nuestros resultados sugieren que la selección sexual debe estar operando sobre los rasgos favorecidos en contextos extra pareja.

6. La distribución espacio-temporal de los individuos determina el balance entre costes y beneficios de seguir una u otra estrategia reproductiva. Sin embargo, en función de la escala de estudio, pueden obtenerse conclusiones diferentes. Una alta sincronía de cría disminuyó el tiempo efectivo para tener EPP a nivel poblacional, pero aumentó las probabilidades de obtenerla para los machos que criaron temprano con respecto a sus vecinos (ver conclusiones **3** y **4**). Espacialmente, la probabilidad de

incurrir en EPP decayó con la distancia entre nidos a nivel poblacional, mientras que las relaciones EPP no se dieron entre los vecinos más cercanos cuando se analizó a la escala a la que tienen lugar las interacciones. Dado que un evento extra pareja emerge de la interacción entre una hembra, el macho social y el macho extra pareja, las conclusiones anteriores recalcan la necesidad de estudiar los contextos sociales a la escala espacio temporal a la que las interacciones ocurren. Concluimos enfatizando que el entendimiento de la conducta a nivel individual es el nivel básico a estudiar para comprender los procesos implicados en la evolución de la paternidad extra pareja.

7. La eficacia biológica, estimada a partir de la supervivencia, no estuvo relacionada con la variabilidad genética (medida con 15 marcadores neutrales). Los individuos más heterocigotos no tuvieron más probabilidades de sobrevivir hasta criar ni vivieron más años que los menos heterocigotos. La falta de relación no fue dependiente del contexto, ya que no varió entre años ni bajo ninguna de las condiciones estudiadas (carga parasitaria del nido, el estatus de la pollada, la fecha de eclosión o la heterocigosidad de los padres). Varias razones pueden explicar la falta de correlación entre supervivencia y heterocigosidad: i) cualquier efecto de la heterocigosidad en la supervivencia podría haber sido contrarrestado por la estocasticidad ambiental que los volantones encuentran tras salir del nido; ii) la presión selectiva en relación a la heterocigosidad podría ocurrir en etapas previas a las analizadas (durante el desarrollo embrionario y el periodo en el nido); iii) los marcadores utilizados podrían no estar relacionados con ningún locus asociado a la supervivencia y/o ser una pobre aproximación a la variabilidad genética a nivel del genoma de los individuos.

8. El resultado anterior reitera la necesidad de estudiar aspectos afines a la eficacia biológica mediante marcadores sujetos a selección. En este sentido, el MHC puede ser un candidato excepcional debido al papel primordial que juega en el sistema

inmune. El protocolo desarrollado en esta tesis permite caracterizar por primera vez genes funcionales de MHC clase II y, a la vez, descartar pseudogenes y genes monomórficos en el papamoscas cerrojillo. Además, queda demostrada su aplicabilidad en otras especies de paseriformes, en los que ha resultado tremendamente complicado hasta la fecha estudiar el MHC debido al gran número de duplicaciones génicas que este sistema suele presentar.

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